(54) Title: METHOD FOR DIAGNOSIS OF LYME DISEASE

(57) Abstract

Methods and reagents are provided for amplifying and detecting target nucleic acid sequences in a sample suspected of containing *Borrelia* spirochetes using the polymerase chain reaction (PCR) process. The PCR process employs primers and probes which are derived from the 16S ribosomal RNA gene of *Borrelia burgdorferi* and *Borrelia hermsii*.

-1-

METHOD FOR DIAGNOSIS OF LYME DISEASE

Cross-Reference to Related Applications

This is a continuation-in-part application of
U.S. Serial No. 07/489,676 filed 7 March 1990, which is
incorporated herein by reference.

Technical Field

The invention relates generally to methods
and reagents for the detection of Lyme disease, a
multisystem bacterial infection transmitted by Ixodid
ticks. More particularly, the methods employ
polymerase chain reaction to amplify genomic nucleic
acid sequences specific to the etiologic agent of Lyme
disease, Borrelia burgdorferi.

Background of the Invention

Lyme disease is a complex multisystem
disorder caused by the tick-borne spirochete Borrelia
burgdorferi. This disease has three clinical stages
that can overlap or occur alone: stage one -- early
disease, including a characteristic expanding skin
lesion (erythema chronicum migrans) and constitutional
flu-like symptoms; stage two -- cardiac and
neurological disease; and stage three -- arthritis and
chronic neurological syndromes.

Presently, the incidence of reported Lyme
disease is increasing, which is probably due to
improved awareness and recognition of the disease, as
well as to an actual increase in incidence and
geographic spread. B. burgdorferi can be isolated from
blood or skin biopsies taken from acutely ill patients,
but the yield is low and the procedures are difficult.

-2-

Serologic testing for antibodies using an enzyme-linked immunosorbent assay (ELISA) or indirect immunofluorescence assay (IFA) is the standard method used to confirm a clinical diagnosis, but current tests are poorly standardized, and false-negative or false-positive results can occur (Barbour, (1989) Ann Intern Med 110:501). In addition to misdiagnosis caused by lack of standardization of serologic testing, cross-reactivity with Treponema and with other Borrelia may occur. Patients with stage one or two disease may be seronegative because it may take as long as three to six months after exposure for antibodies to become detectable with currently available tests. Patients who develop later stages of the illness may occasionally be seronegative if they were treated acutely with antibiotics (Dattwyler et al., (1988) N Engl J Med 319:1441). Previously untreated patients with a late stage of the disease are, apparently, almost always seropositive.

The use of specific polynucleotide sequences as probes for the recognition of infectious agents is becoming a valuable alternative to problematic immunological identification assays. For example, PCT publication WO84/02721, published 19 July 1984 describes the use of nucleic acid probes complementary to targeted nucleic acid sequences composed of ribosomal RNA, transfer RNA, or other RNA in hybridization procedures to detect the target nucleic acid sequence. While the assay may provide greater sensitivity and specificity than known DNA hybridization assays, hybridization procedures which require the use of a complementary probe are generally dependent upon the cultivation of a test organism and are, therefore, unsuitable for rapid diagnosis.

Polymerase chain reaction (PCR) is a powerful technique that can be used for the detection of small

-3-

numbers of pathogens whose in vitro cultivation is difficult or lengthy, or as a substitute for other methods which require the presence of living specimens for detection. In its simplest form, PCR is an in vitro method for the enzymatic synthesis of specific DNA sequences, using two oligonucleotide primers that hybridize to opposite strands and flank the region of interest in the target DNA. A repetitive series of cycles involving template denaturation, primer annealing, and the extension of the annealed primers by DNA polymerase results in the exponential accumulation of a specific fragment whose termini are defined by the 5' ends of the primers. PCR reportedly is capable of producing a selective enrichment of a specific DNA sequence by a factor of 10^9. The PCR method is described in Saiki et al., (1985) Science 230:1350 and is the subject of U.S. Patent Nos. 4,683,195; 4,683,202; 4,800,159; and 4,965,188. This method has been used to detect the presence of the aberrant sequence in the beta-globin gene which is related to sickle cell anemia (Saiki et al., (1985) supra) and the human immunodeficiency virus (HIV) RNA (Byrne et al., (1988) Nuc Acids Res 16:4165). However, before the method can be used, enough of the nucleotide sequence of the disease-associated polynucleotide must be known to design primers for the amplification, and to design probes specific enough to detect the amplified product.

Both genomic and plasmid DNA sequences have been used for the identification of Borrelia, but neither of these methods have been shown to detect all Borrelia burgdorferi isolates. Schwan et al., [(1989) J Clin Microbiol 27:1734 and (1988) Ann NY Acad Sci 539:419] use nucleic acid hybridization probes derived from the 49 kilobase linear plasmid of B. burgdorferi for the detection and identification of B. burgdorferi

−4−

from a number of other <u>Borrelia</u> species in the United
States.   A nucleic acid probe containing the 5' portion
of the variable major protein 7 (<u>vmp</u>7) of <u>B</u>. <u>hermsii</u>
(the causative agent of tick-borne relapsing fever) was
5   also employed in these studies.

One of the inherent limitations of this assay
is its dependency on the identification of plasmid
DNA.   Such identification procedures are inherently
unreliable given that plasmids, over time, are unstable
10   in cultured spirochetes or may be absent from some
natural isolates of the pathogen or clinical specimens.

Rosa and Schwan (1989) <u>J Infect Dis</u>
<u>160</u>(6):1018 used PCR to amplify a target selected from
randomly cloned <u>B</u>. <u>burgdorferi</u> DNA.   While the
15   specificity of the PCR assay suggested a utility for
this technique, not all <u>B</u>. <u>burgdorferi</u> isolates were
detected.   Since it is not known currently whether Lyme
disease is caused by all isolates of <u>B</u>. <u>burgdorferi</u>, it
is essential for accurate diagnosis to demonstrate that
20   all isolates will be detected in any given assay
system.   Furthermore, their system is not sensitive
enough to detect the pathogen in clinical or biological
specimens.

In light of current limitations for the
25   serological identification of Lyme disease, it would be
desirable to provide a rapid and sensitive procedure
for the detection of <u>B</u>. <u>burgdorferi</u> in a clinical
sample suspected of containing the spirochete.   It
would also be desirable to develop reagents that are
30   useful for detecting all geographical isolates of
<u>B</u>. <u>burgdorferi</u>.

<u>Brief Description of the Drawings</u>
FIG. 1 shows a comparison of a partial region
35   of the nucleotide sequences of the 16S rRNA genes from

E. coli (top line - SEQ ID No: 13) aligned for maximum
identity with B. burgdorferi (lower line - SEQ ID No:
1). The region shown corresponds to nucleotides 661
through 1542, numbered in accordance with the
5   nucleotide sequence of the E. coli 16S rRNA gene. The
nucleotides marked by asterisk superscripts are
heterogeneous between the two bacteria.

FIG. 2 is a partial linear representation of
the 16S rRNA gene shown in FIG. 1. The various primers
10   are designated therein with respect to their point of
initiation.

FIG. 3 illustrates a photograph of a 2%
NuSieve plus 0.5% Seakem agarose gel visualized using
ethidium bromide and UV light. This photograph
15   demonstrates amplification of approximately 1.7
femtograms (fg) of B. burgdorferi DNA using the primers
DD02 (SEQ ID No: 2) and DD06 (SEQ ID No: 6).

FIG. 4 is an example of the sensitivity of
detection of B. burgdorferi DNA showing dot blot
20   analysis of the PCR-amplified samples using the DDO4
(SEQ ID No: 4) radiolabeled probe.

FIG. 5 is an photograph of an agarose gel
illustrating the results of a nested PCR
coamplification experiment using external primers DD17
25   (SEQ ID No: 17) and DD18 (SEQ ID No: 18) and internal
primers DD19 (SEQ ID No: 19) and DD20 (SEQ ID No: 20).

FIG. 6 shows a comparison of a partial region
of the nucleotide sequences of the 16S rRNA genes from
B. burgdorferi (top line - SEQ ID No: 1) aligned for
30   maximum identify with B. hermsii (middle line - SEQ ID
No: 29). The bottom line indicates differences in
specific nucleotides.

Disclosure of the Invention
35   The present invention pertains to methods and
reagents useful for the detection of Borrelia species,

-6-

particularly those species known to be the etiologic
agents of Lyme disease and tick-borne relapsing fever.
Detection of the presence of the agent is based upon
the detection of the gene coding for the 16S ribosomal
5   RNA (rRNA), as well as fragments or transcripts derived
therefrom, in biological samples. The method entails
amplifying a target region from genomic DNA or from a
reverse transcript of Borrelia 16S rRNA and detecting
the amplified polynucleotide by hybridization. The
10   method utilizes primers and probes which are available
due to the information provided in portions of the 16S
rRNA gene sequence.

     Studies of the structure of the 16S rRNA gene
sequence from bacterial species suggest that there are
15   regions of conserved sequence among these genes. These
conserved sequences allow the creation of primers which
may be universal in their application for amplification
of target regions of the genus Borrelia.
Identification of the species is then accomplished
20   utilizing a probe specific for the species;
identification of Borrelia burgdorferi is accomplished
utilizing a probe specific for this species, the
sequence of which can be determined from the Borrelia
polynucleotide sequences provided herein.
25   Alternatively, utilization of different hybridization
and wash conditions allows a general detection system
which can detect all Borrelia species.

     It would also be desirable to identify
primers that permit specific amplification of a target
30   region which does not require the use of a probe for
subsequent identification of the target sequence. The
efficiency of this process rests upon the knowledge of
the primer sequences used to amplify the Borrelia DNA.

     Accordingly, one aspect of the invention is a
35   method for determining the presence of a Borrelia

-7-

polynucleotide in a sample suspected of containing said
Borrelia polynucleotide, wherein said Borrelia
polynucleotide comprises a selected target region, said
method comprising:

5   (a) amplifying the target region to a
detectable level, wherein said target region contains a
Borrelia 16S ribosomal polynucleotide sequence;

             (b) providing a polynucleotide probe
containing a sequence which is complementary to the
10   Borrelia 16S ribosomal polynucleotide sequence in the
target region;

             (c) incubating the amplified target
region with the polynucleotide probe under conditions
which allow specificity of hybrid duplexes; and

15   (d) detecting hybrids formed between
the amplified target region and the polynucleotide
probe.

             Another aspect of the invention provides
a method for amplifying a target region in a 16S
20   ribosomal Borrelia polynucleotide comprising:

             (a) providing a set of primers, wherein
a first primer contains a region which is sufficiently
complementary to a sequence in a sense strand of 16S
ribosomal Borrelia polynucleotide to prime the
25   synthesis of a DNA strand which is a complement to the
sense strand of the target region, and a second primer
which contains a region which is sufficiently
complementary to a sequence in an antisense strand of
16S ribosomal Borrelia polynucleotide to prime the
30   synthesis of a DNA strand which is a complement to the
antisense strand of the target region;

             (b) providing a sample suspected of
containing the target region;

             (c) contacting the sample with four
35   different nucleoside triphosphates, the first and

-8-

second primers, and a primer- and template-dependent
polymerizing agent, under conditions whereby the
primers are extended using the target region as
template, yielding primer extension products;

5   (d) treating the primer extension
products under denaturing conditions to separate the
primer extension products from their templates; and

               (e) repeating steps (c) and (d) until
the target sequence is amplified to a level which
10   allows it to be detected above non-target levels in the
sample.

          Yet another aspect of the invention provides
an oligonucleotide probe for detecting or monitoring
Borrelia species nucleic acid in a sample comprising a
15   sequence capable of hybridizing to an amplified 16S
ribosomal RNA of Borrelia or a sequence complementary
thereto.

          The invention also contemplates a polymerase
chain reaction (PCR) kit for amplifying a target region
20   in a Borrelia 16S ribosomal polynucleotide, comprising:

               (a) a first container in which are
stored a set of primers, one for each strand of the
Borrelia nucleic acid sequence to be amplified, which
primers are sufficiently complementary to substantially
25   conserved regions among the Borrelia 16S ribosomal DNA
to hybridize therewith, such that an extension product
synthesized from each primer is substantially
complementary to a strand of the Borrelia DNA sequence
being amplified, such that the extension product
30   synthesized from each primer, when separated from its
complement, can serve as a template for the synthesis
of the extension product of the other primer; and

               (b) one or more additional containers
in which are stored reagents for performing PCR
35   amplification and, optionally, probe hybridization.

-9-

Detailed Description of the Invention

A.   Definitions

As used herein, a "biological sample" refers to a sample of tissue or fluid isolated from an individual, including but not limited to, for example, skin, plasma, serum, spinal fluid, synovial fluid, lymph fluid, urine, blood cells, organs, such as the spleen or the kidney, and also samples of in vitro cell culture constituents.

As used herein, the terms "polynucleotide" and "oligonucleotide" refer to primers, probes, oligomer fragments to be detected, oligomer controls, and unlabeled blocking oligomers and shall be generic to polydeoxy-ribonucleotides (containing 2-deoxy-D-ribose), to polyribonucleotides (containing D-ribose), and to any other type of polynucleotide which is an N-glycoside of a purine or pyrimidine base, or modified purine or pyrimidine bases. There is no intended distinction in length between the term "polynucleotide" and "oligonucleotide", and these terms will be used interchangeably. These terms refer only to the primary structure of the molecule. Thus, these terms include double- and single-stranded DNA, as well as double- and single-stranded RNA.

The term "primer" may refer to more than one primer and refers to an oligonucleotide, whether occurring naturally, as in a purified restriction digest, or produced synthetically, which is capable of acting as a point of initiation of synthesis along a complementary strand when placed under conditions in which synthesis of a primer extension product which is complementary to a nucleic acid strand is catalyzed. Such conditions include the presence of four different deoxyribonucleoside triphosphates and a polymerization-inducing agent such as DNA polymerase or

-10-

reverse transcriptase, in a suitable buffer ("buffer" includes substituents which are cofactors, or which affect pH, ionic strength, etc.), and at a suitable temperature. The primer is preferably single-stranded
5   for maximum efficiency in amplification.

As used herein, the term "target sequence" refers to a region of the polynucleotide which is to be amplified and/or detected.

The term "probe" refers to a polynucleotide
10   or oligonucleotide which forms a hybrid structure with a sequence in a target region, due to complementarity of at least one sequence in the probe with a sequence in the target region. The probe may contain a sequence complementary to either the sense or antisense strand
15   of the target region. Preferably the probe, however, does not contain a sequence complementary to sequence(s) used to prime the polymerase chain reaction.

As used herein, the term "thermostable nucleotide polymerase" refers to an enzyme which is
20   relatively stable to heat when compared to nucleotide polymerases from E. coli and which catalyzes the polymerization of nucleosides. Generally, the enzyme will initiate synthesis at the 3'-end of the target sequence utilizing the primer, and will proceed in the
25   3'-direction along the template until synthesis terminates. A representative thermostable enzyme isolated from Thermus aquaticus (Taq) is described in U.S. Patent No. 4,889,818 and a method for using it is described in Saiki et al., (1988), Science 239:487.

30   An "individual", as used herein, refers to vertebrates, particularly members of the mammalian species, and includes, but is not limited to, domestic animals, sport animals, wild animals, and primates, including humans; and invertebrates such as ticks.

35

-11-

B.   General Method

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology, microbiology and recombinant DNA techniques, which are within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Sambrook, Fritsch & Maniatis, Molecular Cloning; A Laboratory Manual, Second Edition, (1989) (hereinafter "Maniatis"); Oligonucleotide Synthesis (M.J. Gait, ed., 1984); Nucleic Acid Hybridization (B.D. Hames & S.J. Higgins, eds., 1984); A Practical Guide to Molecular Cloning (B. Perbal, 1984); and a series, Methods in Enzymology (Academic Press, Inc.). All patents, patent applications, and publications mentioned herein, both supra and infra, are hereby incorporated by reference.

The various aspects of the invention described herein allows the detection of the etiologic agent of Lyme disease, by virtue of the detection in biological samples of the DNA derived from the bacteria. As used herein, a polynucleotide "derived from" a designated polypeptide sequence refers to a polynucleotide sequence which is comprised of a sequence of approximately at least 6 nucleotides, preferably at least about 10-12 nucleotides, and more preferably at least about 15-20 nucleotides corresponding to a region of the designated nucleotide sequence. "Corresponding" means identical to or complementary to the designated sequence.

The derived polynucleotide is not necessarily physically derived from the Borrelia nucleotide sequence shown in Figure 1, but may be generated in any manner, including chemical synthesis, DNA replication, reverse transcription or a combination thereof. The term "recombinant polynucleotide" intends a

-12-

polynucleotide of genomic DNA or RNA, cDNA, semisynthetic, or synthetic origin which, by virtue of its origin or manipulation: (1) is not associated with all or a portion of the polynucleotide with which it is associated in nature; and/or (2) is linked to a polynucleotide other than that to which it is linked in nature; and (3) is not found in nature.

The methods of the present invention enable one to obtain greater sensitivity and accuracy than heretofore possible with prior art detection methods for Borrelia. The basic PCR process is carried out as follows.

A sample is provided which is suspected of containing a particular nucleic acid sequence of interest, the "target sequence". The nucleic acid contained in the sample may be first reverse transcribed into cDNA, if necessary, and then denatured, using any suitable denaturing method, including physical, chemical, or enzymatic means, which are known to those of skill in the art. A preferred physical means for strand separation involves heating the nucleic acid until it is completely (>99%) denatured. Typical heat denaturation involves temperatures ranging from about 80°C to about 105°C, for times ranging from about 1 to 10 minutes.

The denatured DNA strands are then incubated with the selected oligonucleotide primers under hybridization conditions, conditions which enable the binding of the primers to the single oligonucleotide strands. As known in the art, the primers are selected so that their relative positions along a duplex sequence are such that an extension product synthesized from one primer, when it is separated from its complement, serves as a template for the extension of the other primer to yield a replicate chain of defined length.

-13-

The primer must be sufficiently long to prime the synthesis of extension products in the presence of the agent for polymerization. The exact length of the primers will depend on many factors, including temperature, source of the primer and use of the method. For example, depending on the complexity of the target sequence, the oligonucleotide primer typically contains about 15-35 nucleotides, although it may contain more or fewer nucleotides. Short primer molecules generally require cooler temperatures to form sufficiently stable hybrid complexes with the template. The primers must be sufficiently complementary to selectively hybridize with their respective strands.

The primers used herein are selected to be "substantially" complementary to the different strands of each specific sequence to be amplified. The primers need not reflect the exact sequence of the template, but must be sufficiently complementary to selectively hybridize with their respective strands. Non-complementary bases or longer sequences can be interspersed into the primer, provided that the primer retains sufficient complementarity with the sequence of one of the strands to be amplified to hybridize therewith, and to thereby form a duplex structure which can be extended by the polymerizing means. The non-complementary nucleotide sequences of the primers may include restriction enzyme sites. Appending a restriction enzyme site to the end(s) of the target sequence is particularly helpful for subsequent cloning of the target sequence.

It is anticipated that there may be a number of strains or geographic isolates of Borrelia with sequences which deviate from the composite sequence provided in Figure 1. Therefore, in order to detect

-14-

such variant strains it is preferable to construct primers which hybridize to conserved regions of the <u>Borrelia</u> <u>burgdorferi</u> 16S rRNA gene sequence. The conserved regions may be determined by comparing the

5   nucleotide sequences of several <u>Borrelia</u> strains or isolates essentially as shown in FIG. 6.

The oligonucleotide primers and probes may be prepared by any suitable method. Methods for preparing oligonucleotides of specific sequence are known in the

10   art, and include, for example, cloning and restriction of appropriate sequences, and direct chemical synthesis. The primers may be labeled, if desired, by incorporating means detectable by spectroscopic, photochemical, biochemical, immunochemical, or chemical

15   means.

Template-dependent extension of the oligo-nucleotide primer(s) is then catalyzed by a polymerizing agent in the presence of adequate amounts of the four deoxyribonucleoside triphosphates (dATP,

20   dGTP, dCTP, and dTTP) or analogs, in a reaction medium which is comprised of the appropriate salts, metal cations, and pH buffering system. Suitable polymerizing agents are enzymes known to catalyze primer- and template-dependent DNA synthesis. Known

25   DNA polymerases include, for example, <u>E. coli</u> DNA polymerase I or its Klenow fragment, T_4 DNA polymerase, and <u>Taq</u> DNA polymerase. The reaction conditions for catalyzing DNA synthesis with these DNA polymerases are well known in the art.

30   The products of the synthesis are duplex molecules consisting of the template strands and the primer extension strands, which include the target sequence. These products, in turn, serve as templates for another round of replication. In the second round

35   of replication, the primer extension strand of the

-15-

first cycle is annealed with its complementary primer; synthesis yields a "short" product which is bounded on both the 5'- and the 3'-ends by primer sequences or their complements. Repeated cycles of denaturation, primer annealing, and extension result in the exponential accumulation of the target region defined by the primers. Sufficient cycles are run to achieve the desired amount of polynucleotide containing the target region of nucleic acid. The desired amount may vary, and is determined by the function which the product polynucleotide is to serve.

The PCR method can be performed in a number of temporal sequences. For example, it can be performed step-wise, where after each step new reagents are added, or in a fashion where all of the reagents are added simultaneously, or in a partial step-wise fashion, where fresh reagents are added after a given number of steps.

In a preferred method, the PCR reaction is carried out as an automated process which utilizes a thermostable enzyme. In this process the reaction mixture is cycled through a denaturing step, a primer annealing step, and a synthesis step. A DNA thermal cycler specifically adapted for use with a thermostable enzyme may be employed, which utilizes temperature cycling without a liquid-handling system, thereby eliminating the need to add the enzyme at every cycle. This type of machine is commercially available from Perkin-Elmer Cetus Instruments and is described in European Patent publication 236,069 published 9 September 1989.

Nested PCR may be performed by using both external primers and "nested" primers. These nested primers define sequences within the target sequence amplified by the external primers. The external

-16-

primers are generally used to preferentially amplify a specific target. During a second round of amplification, the PCR conditions are designed such that the internal primers preferentially amplify the
5   target region within the product amplified by the external primers. These experiments usually employ sets of primers that have different melting temperatures (Tm). For example, if the external primers have a higher Tm than the internal primers, the
10   annealing temperature used in the first round of amplification is set higher, generally about 50 to 65°C. In addition, the concentrations of the internal primers are generally in excess over the external primers. A ratio of 50 pmoles:5 pmoles is usually
15   sufficient. As the annealing temperature is lowered to, for example, about 35 to 50°C for the second round of the amplification, preferential amplification of the fully nested product occurs due in part to the limited concentration of the external primers.

20   The nested system may also be performed using a hemi-nested primer, that is only a single internal primer is used during the second amplification along with the appropriate "external" primer from the first amplification cycle. If this system in employed,
25   appropriate adjustment of the concentration of the dual purpose external primer must be made.

            The advantage of these systems is two-fold: 1) the sensitivity of a system can be increased by allowing amplification of a low copy input target
30   sequence; and 2) the specificity of a system can be increased by eliminating amplification of nonspecific sequences. Only sequences which are defined by both the internal and the external primers will be amplified using nested PCR.

35   After amplification by PCR, the target polynucleotides may be detected directly by gel

-17-

analysis provided the target DNA is efficiently
amplified and the primers are highly specific to the
target region to be amplified. To assure PCR
efficiency, glycerol and other related solvents such as
5   dimethyl sulfoxide, can be used to increase the
sensitivity of the PCR at the amplification level and
to overcome problems pertaining to the sequencing of
regions of DNA having strong secondary structure.
These problems may include: (1) low efficiency of the
10   PCR, due to a high frequency of templates that are not
fully extended by the polymerizing agent or
(2) incomplete denaturation of the duplex DNA at high
temperature, due to high GC content. The use of such
solvents increases the sensitivity of the assay at the
15   level of amplification to approximately several
femtograms of DNA (which is believed to correspond to a
single spirochete cell). This level of sensitivity
eliminates the need to detect amplified target DNA
using a probe, and thereby dispenses with the
20   requirements for radioactive probes, gel
electrophoresis, Southern blotting, filter
hybridization, washing and autoradiography. The
concentration range for glycerol is about 5%-20% (v/v),
with 10% being preferred, and the DMSO concentration
25   range is about 3% - 10% (v/v).

          Alternatively, the target polynucleotides may
be detected by hybridization with a probe
polynucleotide which forms a stable hybrid with that of
the target sequence under stringent to low stringency
30   hybridization and wash conditions. If it is expected
that the probes will be completely complementary (i.e.,
about 99% or greater) to the target sequence, stringent
conditions will be used. If some mismatching is
expected, for example if variant strains are expected
35   with the result that the probe will not be completely

complementary, the stringency of hybridization may be
lessened. However, conditions are chosen which rule
out nonspecific/adventitious binding. Conditions which
affect hybridization and which select against
5   nonspecific binding are known in the art and are
described 'in, for example, Maniatis et al. (1989).
Generally, lower salt concentration and higher
temperature increase the stringency of binding. For
example, it is usually considered that stringent
10   conditions are incubation in solutions which contain
approximately 0.1 X SSC, 0.1% SDS, at about 65°C
incubation/wash temperature, and moderately stringent
conditions are incubation in solutions which contain
approximately 1-2 X SSC, 0.1% SDS and about 50°-65°C
15   incubation/wash temperature. Low stringency conditions
are 2 X SSC and about 30°-50°C.

        Probes for Borrelia target sequences may be
derived from either strand of the 16S rRNA DNA
sequences described infra, or from new Borrelia
20   isolates. The Borrelia probes may be of any suitable
length which span the target region, but which exclude
the primers, and which allow specific hybridization to
the target region. If there is to be complete
complementarity, i.e., if the strain contains a
25   sequence identical to that of the probe, since the
duplex will be relatively stable under even stringent
conditions, the probes may be short, i.e., in the range
of about 10-30 basepairs. If some degree of mismatch
is expected with the probe, i.e., if it is suspected
30   that the probe will hybridize to a variant region, the
probe may be of greater length, since length seems to
counterbalance some of the effect of the mismatch(es).

        In certain embodiments of the invention,
probes have been designed to permit the detection of
35   specific species of Borrelia. With reference to FIG.
2, the probe DDO4 (SEQ ID No: 4)

5'-CCCGNAAGGGAGGAAGGTATN-3' wherein N at position 5 can be T or C and N at position 21 can be T, C or zero, or the sequence complementary thereto (antisense probe DD14 (SEQ ID No: 14)), can distinguish B. burgdorferi species over other Borrelia species using stringent hybridization and wash conditions when N at position 5 is T and N at position 21 is absent. B. hermsii, on the other hand, may be detected when N at position 5 is C and N at position 21 is either C or absent. Once the sequence of the 16S ribosomal gene of the particular species or strain to be detected is determined using the method of the present invention, one of ordinary skill in the art can design the appropriate probes to detect the particular target of interest.

If desired, the probe may also be labeled. A variety of labels which would be appropriate, as well as methods for their inclusion in the probe are known in the art, and include, for example, 32-radioactive atoms, such as ^{32}P, or other recognizable functionalities, e.g., biotin (preferably using a spacer arm), fluorescent dyes, electron-dense reagents, enzymes capable of forming easily detectable reaction products (e.g., alkaline phosphatase, and horseradish peroxidase), or antigens for which specific antisera or monoclonal antibodies are available.

In order to determine variant Borrelia species or isolates, and thereby to design probes for those variants, the above-described PCR method is utilized to amplify variant regions of the Borrelia 16S rRNA gene, so that the nucleotide sequences of these variant target regions can be determined. Generally, variant types of Borrelia are known to occur in different geographic locations, for example, Europe and North America, including Mid-America, New England, North Central, Southeast, Southwest, Mountain and

-20-

Pacific regions; or in different vertebrate species which are infected with the spirochete.

In order to amplify the variant target region, primers are designed to flank the suspect region, and preferably are complementary to substantially conserved regions of at least 12 nucleotides long in the 16S rRNA gene. The probe is designed for the variant region with some degree of mismatch expected. Analysis of the nucleotide sequence of the target region may be by direct analysis of the PCR amplified products as described in Gyllensten and Erlich, (1988) Proc Natl Acad Sci USA 85:7652 and in PCT publication WO90/03444, published 5 April 1990.

It may be desirable to determine the length of the PCR product detected by the probe. This may be particularly true if it is suspected that variant Borrelia strains may contain deletions within the target region, or if one wishes to confirm the length of the PCR product. In such circumstances, it is preferable to subject the products to size analysis as well as hybridization with the probe. Methods for determining the size of nucleic acids are known in the art, and include, for example, gel electrophoresis, sedimentation in gradients, and gel exclusion chromatography.

The presence of the target sequence in a biological sample is detected by determining whether a hybrid has been formed between the Borrelia probe and the nucleic acid subjected to the PCR amplification techniques. Methods to detect hybrids formed between a probe and a nucleic acid sequence are known in the art. For example, an unlabeled sample may be transferred to a solid matrix to which it binds, and the bound sample subjected to conditions which allow specific hybridization with a labeled probe; the solid

matrix is then examined for the presence of the labeled probe. Alternatively, if the sample is labeled, an unlabeled probe is bound to the matrix, and after exposure to the appropriate hybridization conditions,
5   the matrix is examined for the presence of a label. PCT publication WO89/11548, pulished 30 November 1989 and Saiki et al., (1988) Proc Natl Acad Sci, USA 86:6230 describe methods of immobilizing multiple probes on a solid support and using hybridization to
10   detect the amplified target polynucleotides of interest. In another alternative procedure, a solution phase sandwich assay may be used with labeled polynucleotide probes, and the methods for the preparation of such probes are described in U.S. Patent
15   No. 4,820,630, issued 11 April 1989.

        C.   PCR Kit and Reagents
            Also within the scope of the present invention is a PCR kit for use in carrying out any of
20   the aforementioned PCR processes. The diagnostic kits include the probe polynucleotide(s) and the primers in separate containers. Either of these may or may not be labeled. If unlabeled, the ingredients for labeling may also be included in the kit. The kit may also
25   contain other suitably packaged reagents and material needed for the particular hybridization protocol, for example, standards, and/or polymerizing agents, as will as instructions for conducting the test.
            In use, the components of the PCR kit, when
30   applied to a nucleic acid sample, create a reagent mixture which enables the detection and amplification of the target nucleic acid sequence. The reagent mixture thus includes the components of the kit as well as a nucleic acid sample which contains the
35   polynucleotide chain of interest.

-22-

The following examples are intended to be illustrative of the various methods and compounds of the invention.

5   EXAMPLES

Example 1. DNA Isolation

Borrelia cultures were maintained in BSK II medium (Barbour (1984) Yale J Biol Med 57:71) at 37°C and passaged twice a week. The species and strains 10 propagated for this study included B. burgdorferi from I. dammini (ATCC 35210), B. burgdorferi from I. ricinus (ATCC 35211), and B. hermsii from O. hermsii (ATCC 35209).

Total genomic Borrelia DNA was prepared as 15 follows. Briefly, spirochetes were pelleted from 100 ml of a stationary-phase culture and resuspended in 0.5 ml total volume of 446 ul of TE (stock solution = 1 mM Tris-HCl, .01 mM EDTA, pH 8.0), 5 ul of 10 mM DTT, 20 ul of 10 mM EDTA, 25 ul of 0.5% SDS and 3.8 ul of 20 Proteinase K (150 ug/ml). The sample was incubated at 37°C for 1 hr. The sample was subsequently extracted twice with phenol-chloroform (1:1) and the upper layer re-extracted. The organic lower layer was also extracted twice with phenol-chloroform and added to the 25 aqueous layer from above. Nucleic acid was precipitated by adjusting the salt concentration of 0.3 M sodium acetate and by the addition of 0.6 volumes of isopropanol. The precipitated DNA was pelleted from the solution, washed once with 0.5 ml of 70% ethanol, 30 and resuspended in 50 ul of TE (stock solution) overnight at 4°C to solubilize the DNA. This sample was then digested with RNase A (100 ug/ml) at 37°C for 1 hr and then extracted twice with phenol/chloroform, precipitated, and resuspended in 25 ul of TE as 35 described above. DNA concentration was determined by optical density.

-23-

Example 2. PCR Amplification of 16S Ribosomal DNA
          Oligonucleotide primers, based upon bacterial
16S rDNA sequence information, were prepared by either
the phosphotriester method as described by Duckworth et
5   al., (1981) Nuc Acids Res 9:1691 or by the
phosphoramidite method as described by Beaucage and
Caruthers, (1981) Tet Letts J Am Chem Soc 22:1859, and
Matteucci and Caruthers, (1981) J Am Chem Soc 103:3185,
and were prepared using commercially available
10   automated oligonucleotide synthesizers, such as the
Applied Biosystems 380A DNA synthesizer. The upstream
and downstream primers used to amplify and partially
sequence the B. burgdorferi 16S ribosomal RNA gene are
listed below, along with the corresponding nucleotide
15   position according to the numbering of the E. coli 16S
ribosomal RNA gene sequence:

          PL3   (5'-ACAAGCGGTGGAGCATGTGG-3')   934->953
                 (SEQ ID No: 25)
20
          PL6   (5'-GGTTAAGTCCCGCAACGAGCGC-3')   1087->1108
                 (SEQ ID No: 26)

          DG73   (5'-TACGTTCCCGGGCCTTGTAC-3')   1375->1394
25   (SEQ ID No: 27)

          DG74   (5'-AGGAGGTGATCCAACCGACA-3')   1522<-1540
                 (SEQ ID No: 28)

30   The arrow position designates the direction of
synthesis.

          Asymmetric PCR was performed according to the
procedure of Gyllensten ("Direct Sequencing of In Vitro
35   Amplified DNA" in PCR Technology, Principles and
Applications for DNA Amplification, ed. Erlich,
Stockton Press 1989) in which the molar ratios of the
primers utilize first one primer in excess
concentration (50 pmoles of DG73 - SEQ ID No: 27) with

-24-

the other primer in limiting concentration (1 pmole of
DG74 - SEQ ID No: 28) in a 100 ul PCR. The reaction is
also performed with the molar ratios of the primers
reversed. In general, a ratio of 50 pmol:0.5 pmol for
5   a 100 ul PCR reaction will result in about 1-3 pmol of
single-stranded DNA (ssDNA) after 30 cycles of PCR.
The recommendation in Gyllensten to reduce the
concentration of each dNTP for asymmetric PCR was
followed. Approximately 62.5 uM of each dNTP was used
10   for a 100 ul PCR reaction buffer.

The ssDNA generated was purified for
sequencing using Amicon-Centricon 30 concentrator
tubes. The purified DNA was then sequenced using an
alpha-[^{35}S]thio dATP, limiting primer and applying
15   conventional protocols for sequencing using Taq DNA
polymerase (see, for example, "Sequencing with Taq DNA
Polymerase" in PCR Protocols, A Guide to Methods and
Applications, ed. Innis et al., Academic Press, Inc.
1990). The products were resolved on buffer gradient
20   sequencing gels.

From this reaction, approximately 84
nucleotides of Borrelia DNA were sequenced,
corresponding to nucleotides 1409 to 1493, designated
in Figure 1. In separate PCR reactions, two different
25   upstream primers (PL3 - SEQ ID No: 25 and PL6 - SEQ ID
No: 26) designed to overlap the same region of the
target DNA from the same organism, were used to verify
the sequence previously obtained. The homology for the
region sequenced was 100%.

30   The B. burgdorferi sequence obtained from
this experiment was aligned for maximum identity with
nucleotides 661 through 1542 of the E. coli 16S rRNA
gene sequence as shown in FIG. 1. As depicted in this
figure, the numbering of the Borrelia sequences
35   (designated as aligning with the E. coli 16S rRNA gene)

-25-

includes deleted, additional and substituted bases as would be clear to one of skill in the art. The asterisks mark bases heterogeneous between the two genera. Bases designated as "N" could not be determined from the sequencing gel. However, if it is desired to design a primer or internal probe which is to be aligned in the region containing any "N," thymine would be a preferred substitute, unless this region contains a base which is conserved among bacterial 16S rRNA genes.

This sequence information was used to design new primers which amplified _Borrelia_ species but not other common pathogens, e.g., _E. coli_, _S. aureus_ and _P. aeruginosa_. The new primers include those designated below and their positions, numbered in accordance with the _E. coli_ 16S ribosomal RNA gene sequence as shown in FIG. 2.

```
DD02 (3'-CCCTCACTAAACATACCT-5')            (SEQ ID No: 2)
DD03 (5'-CACCCGAGTTGAGGATACC-3')           (SEQ ID No: 3)
DD05 (5'-ATAGTTAGAGATAATTATTC-S')          (SEQ ID No: 5)
DD06 (5'-ATCTGTTACCAGCATGTAAT-3')          (SEQ ID No: 6)
DD07 (5'-CGTGCTACAATGGCCTG-3')             (SEQ ID No: 7)
DD08 (5'-GGAAGATAGTTAGAGATAATTATTC-3')     (SEQ ID No: 8)
DD09 (5'-CTTGTTATCTGTTACCAGCATGTAAT-3')    (SEQ ID No: 9)
DD10 (5'-TATCTGTTACCAGCATGTAAT-3')         (SEQ ID No: 10)
DD11 (5'-ACACACGTGCTACAA-3')               (SEQ ID No: 11)
DD12 (5'-CAGATAAGACTGCCGGTGATAAGTC-3')     (SEQ ID No: 12)
```

Primers DD02 (SEQ ID No: 2) and DD03 (SEQ ID No: 3) were used to amplify _Borrelia_ DNA in a 3-step cycling PCR reaction. To 100 ul of the PCR buffer, including 62.5 um of each dNTP, 5 units/100 ul of _Taq_ DNA polymerase, 2.4 mM $MgCl_2$, were added equimolar concentrations (50 pmoles) of primers DD02 (SEQ ID No: 2) and DD03 (SEQ ID No: 3) to approximately 1.7×10^7 fg of the target DNA. Each resulting solution was heated first to 95°C for 5 min followed by 25 cycles of a denaturing step at 95°C for 25 sec, an annealing step

-26-

at 50°C for 25 sec and an extension step at 72°C for 2 min. A final extension step was performed at 72°C for 10 min.

Purified DNAs from 25 species of medically significant bacteria including 16 spirochetes from the <u>Treponema</u> and <u>Leptospira</u> genera were amplified by the PCR assay as taught above. Since the results indicated that these conditions were not completely specific for <u>Borrelia</u>, a second cycling format was tested. The PCR cycling was increased to 35 cycles and the annealing temperature was raised to 60°C for 25 sec. As shown in Table 1, amplification under these conditions was observed only for <u>B</u>. <u>burgdorferi</u>, <u>B</u>. <u>hermsii</u> and <u>T</u>. <u>pectinovorum</u>.

In addition to modifying the annealing temperature, a third format was developed to reduce the overall assay performance time. In this format, a two-step cycling reaction wherein the denaturing step is performed at 95°C for 25 sec and the annealing step is performed at 60°C for 25 sec, for 30 cycles, was tested. This 2-step PCR format proved to provide a similar level of specificity as that observed in the second format, 3-step cycling reaction, and was therefore adopted for all subsequent PCR assays.

-27-

Table 1

Summary of Lyme Disease Specificity Results

Organism	Strain ATCC/Other	Primers DD2/DD3	Probe DD4	Source
BORRELIA SP.				
burgdorferi	35211	+	+	
burgdorferi	35210	+	+	
burgdorferi	Y297[a]	+	+	
burgdorferi	YN40[a]	+	+	
burgdorferi	CA4-3[b]	+	+	
burgdorferi	CA7-7[b]	+	+	
hermsii	35209	+	-	
TREPONEMA SP.				
denticola	33520	-	-	Subgingival plaque
denticola	35404	-	-	Human perio- dontal pocket
pallidum	27087	-	-	Human syphilis
pectinovorum	33768	+	-	Subgingiva
socranskii sep. buccale	35534	-	-	Subgingival plaque of patient with periodontitis
socranskii sep. paredis	35535	-	-	Subgingival plaque of patient with periodontitis
socranskii sep. socranskii	35536	-	-	Subgingival plaque of patient with periodontitis
vincentii	35580	-	-	Patient with periodontitis
LEPTOSPIRA SP.				
inadi sv. lyme	43289	-	-	Human skin
interrogans st. autumnalis	23476	-	-	Human

-28-

Table 1 (con't)

Summary of Lyme Disease Specificity Results

Organism	Strain ATCC/Other	Primers DD2/DD3	Probe DD4	Source
interrogans st. budapest	23581	-	-	Human
interrogans st. ictero-haemorrhagiae	43642	-	-	Human blood
weilii sv. celledoni	43285	-	-	Human blood
ADDITIONAL BACTERIA				
N.gonorrhoeae		-	-	
S.typhi		-	-	
E.coli		-	-	
P.aeruginosa		-	-	
S.aureus		-	-	
K.pneumoniae		-	-	
S.marcescens		-	-	
E.aerogenes		-	-	
S.pneumoniae		-	-	

[a] = Strains provided by David Persing at Yale University
[b] = Strains provided by Robert Lane at the University of
California at Berkeley [Lane et al., (1989) J Clin Microbiol
27:2344]

The same group of organisms listed in Table 1
and additional strains of B. burgdorferi and other
Borrelia species were also tested in the 2-step PCR
format to test for the specificity of primers DD02 (SEQ
ID No: 2) and DD06 (SEQ ID No: 6) for Borrelia DNA.
One modification to the 2-step format employed a
decrease in the anneal temperature to 55°C from 60°C.
The results of this experiment were generally 100%
specific for B. burgdorferi as none of the other
spirochetes or eubacteria were amplified except for L.
interrogans st. icterohaemorrhagiae. However, the
amplification system is species specific when used in
conjunction with probe hybridization.

-29-

Table 2

Additional Strains Tested

Organism	Strain ATCC/Other	Primers DD2/DD6	Probe DD4	Source
BORRELIA SP				
burgdorferi	35211	+	+	
burgdorferi	35210	+	+	
burgdorferi	Y297[a]	+	+	
burgdorferi	YN40[a]	+	+	
burgdorferi	CA4-3[b]	+	+	
burgdorferi	CA7-7[b]	+	+	
burgdorferi	MEN ZN-6-88	+	+	I. pacificus
burgdorferi	HUM 78-1-4	+	+	I. pacificus
burgdorferi	LAKE 3-3-9	+	+	I. pacificus
burgdorferi	SC 5-2-89	+	+	I. pacificus
burgdorferi	BUCO 2-10-89	+	+	I. pacificus
burgdorferi	SON 3-3-5	+	+	I. pacificus
burgdorferi	CONN 2591	+	+	I. pacificus
burgdorferi	NEV 3-2-88	+	+	I. pacificus
burgdorferi	SON 1-88	+	+	I. pacificus
burgdorferi	DN 1-2-7	+	+	I. pacificus
burgdorferi	FRE 501-3-5	+	+	I. pacificus
burgdorferi	TRI 5-2-89	+	+	I. pacificus
burgdorferi	WISC 21644	+	+	I. pacificus
burgdorferi	TUO 1-3-89	+	+	I. pacificus
burgdorferi	Millbrook 25015	+	+	I. pacificus
burgdorferi	53899	+	+	Human CSF
BORRELIA SP.				
hermsii	35209	+	−	
hermsii	Conrad	+	−	
hermsii	HSI	+	−	
hermsii	York	+	−	
hermsii	4229	+	−	
coriaceae		+	−	
parkeri		+	−	
turicatae		+	−	
anserina		+	−	

-30-

Table 2 (Con't)

Additional Strains Tested

Organism	Strain ATCC/Other	Primer DD2/DL	Probe DD4	Source
TREPONEMA SP.				
denticola	33520	-	-	Subgingival plaque
denticola	35404	-	-	Human perio-dontal pocket
pallidum	27087	-	-	Human syphilis
pectinovorum	33768	-	-	Subgingiva
socranskii sep. buccale	35534	-	-	Subgingival plaque of patient with periodontitis
socranskii sep. paredis	35535	-	-	Subgingival plaque of patient with periodontitis
socranskii sep. socranskii	35536	-	-	Subgingival plaque of patient with periodontitis
vincentii	35580	-	-	Patient with periodontitis
		•		
LEPTOSPIRA SP.				
inadi sv. lyme	43289	-	-	Human skin
interrogans st. autumnalis	23476	-	-	Human
interrogans st. budapest	23581	-	-	Human
interrogans st. ictero-haemorrhagiae	43642	+	-	Human blood
weilii sv. celledoni	43285	-	-	Human blood
ADDITIONAL BACTERIA				
N.gonorrhoeae		-	-	
S.typhi		-	-	
E.coli		-	-	
P.aeruginosa		-	-	

-31-

Table 2 (Con't)

Additional Strains Tested

Organism	Strain ATCC/Other	Primers DD2/DD6	Probe DD4	Source
S.aureus		-	-	
K.pneumoniae		-	-	
S.marcescens		-	-	
E.aerogenes		-	-	
S.pneumoniae		-	-	

a and b - See Table 1.

Example 3. Specificity of PCR Assay

Specific detection of B. burgdorferi was accomplished using hybridization of a radioactively labeled oligonucleotide probe to amplified DNA analyzed by agarose gel electrophoresis and Southern transfer.

The probe DD04 (CCCGTAAGGGAGGAAGGTAT) (SEQ ID No: 4) was labeled with ^{32}P-ATP and T_4 polynucleotide kinase following standard procedures. The unincorporated nucleotides were separated from the incorporated label using a NucTrap^{TM} push column (Stratagene) according to the manufacturer's instructions.

About 5 ul of each amplified PCR product were resolved on a 2% NuSieve/0.5% Seakem agarose gel and visualized by ethidium bromide fluorescence. Hybridization conditions for Southern analysis employed 5X SSPE, 5X Denhardt's solution and 0.1% SDS. The membrane was prehybridized in the buffer for 2 hrs at 60°C prior to the addition of about 70 ul of the DD04 labeled probe. Filters were hybridized at 60°C overnight and then washed in 5X SSPE, 0.1% SDS at room temperature for 10 min, followed by 2 washes in 2X SSPE, 0.1% SDS at 60°C for 15 min and a last wash step in 2X SSPE, 0.1% SDS at 60°C for 30 min. These

-32-

stringent conditions were effective to eliminate any signal from B. hermsii.

As shown in Tables 1 and 2, the use of the internal probe DD04 (SEQ ID No: 4) offers an assay that
5   is 100% sensitive and specific for B. burgdorferi.

Example 4. Species Specificity Using Primers DD06 and DD02

Amplification of the B. burgdorferi target
10   DNA was tested in a PCR with primers DD02 (SEQ ID No: 2) and DD06 (SEQ ID No: 6) in the presence of glycerol and increasing cycling conditions to monitor the sensitivity of the reaction. A 100 ul PCR assay was run using purified B. burgdorferi DNA in varying
15   concentrations (1.7 x 10^0 through 1.7 x 10^3 fg); 50 pmol of each primer; 1.5 mM $MgCl_2$; 62.5 uM of each dNTP/100 ul reaction; 5 units of Taq DNA polymerase/100 ul reaction; and 0 to 15% glycerol (v/v).

The resulting solutions were heated first to
20   95°C for 5 min, followed by 30 to 55 cycles of PCR (increasing at five cycle increments) using the following temperature profile:

Denaturation   95°C, 25 sec
Annealing   55°C, 25 sec
25   A final extension step was performed at 72°C for 10 min. The products of these reactions were separated by electrophoresis using a 2% NuSieve/0.5% Seakem agarose gel, stained with ethidium bromide and visualized by UV light. A photograph of this gel is shown in FIG.3.
30   FIG. 3 provides 3 different gels, each gel representing two different cycling conditions. FIG. 3A was run for 30 cycles, FIG. 3B for 35 cycles, FIG. 3C for 40 cycles, FIG. 3D for 45 cycles, FIG. 3E for 50 cycles and FIG. 3F for 55 cycles. The samples loaded
35   onto Lanes 1 through 24 remained the same throughout

-33-

the three experiments, varying only by the number of
PCR cycles performed and the amount of glycerol present
(designated in FIG. 3). Lanes 1 and 24 are molecular
weight markers of ϕX174 cut with HaeIII (New England
5   Biolabs); Lanes 2 and 23 are blank; Lanes 3, 8, 13 and
18 contain 1.7 x 10^3 fg/100 ul of B. burgdorferi DNA;
Lanes 4, 9, 14 and 19 contain 1.7 10^2 fg/100 ul of B.
burgdorferi DNA; Lanes 5, 10, 15 and 20 contain 1.7 x
10^1 fg/100 ul of B. burgdorferi DNA; Lanes 6, 11, 16
10   and 21 contain 1.7 x 10^0 fg/100 ul of B. burgdorferi
DNA; and Lanes 7, 12, 17 and 22 are negative controls
containing 50 ul H_2O and 50 ul of the PCR cocktail.
     The results show that the presence of
glycerol increased the sensitivity of the PCR by one
15   hundred-fold. By forty cycles (FIG. 3C, Lane 11) the
sensitivity is at the one bacterium level (1.7 fg), and
is very noticeable by 45 cycles (FIG. 3D, Lanes 11, 16
and 21). At 50 and 55 cycles the band at the one
bacterium level is prominent at all concentrations of
20   glycerol. In the absence of glycerol amplification can
be seen at 1.7 x 10^2 fg (FIG. 3A, B, C, Lane 4). As
the cycle number increases the samples without glycerol
deteriorate into smears. The addition of glycerol
appears to maintain the integrity of the PCR reaction
25   at high cycle numbers.

Example 5. Probe Experiments
     The following experiment was designed to test
whether the sensitivity of the PCR assay could be
30   increased by detecting the amplified Borrelia target
sequence with a gene probe.
     About 15 ul of the PCR product was denatured
by incubation with 15 ul of a denaturant (0.8 M NaOH,
50 mM EDTA) for 3 min at room temperature. About 20 ul
35   of the denatured PCR product (using either a 40 or 55

-34-

cycle reaction run in the presence or absence of 10%
glycerol -- see Example 4) was spotted onto a 0.45
micron nylon membrane (Genetrans; Plasco, Inc.), and
fixed onto the membrane by UV irradiation.

5   The amplified DNA immobilized on the nylon
membrane was prehybridized with a hybridization
solution containing 5 ml of 20x SSPE, pH 7.4, 0.2 ml of
10% SDS, 1 ml of Denhardt's solution and 13.8 ml of H_2O
for 1 hr at 50°C.

10   About 70 ul of the kinased probe DD04 (SEQ ID
No: 4) (see Example 3) were added to the membrane in
solution and hybridization was run for 3-5 hrs at
50°C. The membrane was then washed in 5x SSPE, 0.1%
SDS at room temperature for 10 min, then at 50°C for 10
15 min, followed by a wash in 2x SSPE, 0.1% SDS at 50°C
for 10 min. To detect ^{32}P-labeled DNAs, the blots were
covered with Saran Wrap and exposed to X-ray film at
-70°C for 4 hrs.

           The results are shown in the autoradiograph
20 illustrated in FIG. 4. Columns A and B represent 40
and 55 cycles, respectively, of B. burgdorferi DNA
amplified in the absence of glycerol and Columns C and
D represent 40 and 55 cycles, respectively, of B.
burgdorferi DNA amplified in the presence of 10%
25 glycerol. The various amounts of B. burgdorferi DNA
present in each blot are presented in the figure in
descending order (1.7 x 10^3 fg, 1.7 x 10^2 fg, 1.7 x 10^1
fg, and 1.7 x 10^0 fg), in each column along with
appropriate negative controls (bottom line). The
30 results indicate that amplification of the gene
sequence by PCR employing glycerol, coupled with the
gene probe, allows one to detect the target in 1.7 fg
of total DNA in either the 40- or 55-cycled amplified
product. In contrast, the probe can only detect target
35 DNA amplified after 40 and 55 cycles in the absence of

glycerol at the 1.7 x 10^2 fg level, a 100-fold decrease
in sensitivity.

The following probes, derived from the region
including nucleotides 1443 to 1474 of FIG. 1 have been
5   designed to test the specificity of detection among
several species of Borrelia. Each of these probes
detected all Borrelia species under low stringency
hybridization and wash conditions. At minimum, a probe
comprising, for example, the core sequence
10 5'-CCGNAAGGGAGG-3' wherein N is. T or C is useful for
detection of Borrelia species.

SENSE (5'-3')
DD04   CCCGTAAGGGAGGAAGGTAT   (SEQ ID No:4)
15 DD15   TAACCCGTAAGGGAGGA   (SEQ ID No:15)
DD21   AACCCGTAAGGGAGGAAGGTATTTAAG (SEQ ID No:21)
DD23   TTCTAACCCGTAAGGGAGGA   (SEQ ID No:23)
B. hermsii   CCCGCAAGGGAGGAAGGTATC   (SEQ ID No:4)

20 ANTISENSE (3'-5')
DD14   ATACCTTCCTCCCTTACGGG   (SEQ ID No:14)
DD16   TCCTCCCTTACGGGTTA   (SEQ ID No:16)
DD22   CTTAAATACCTTCCTCCCTTACGGGTT (SEQ ID No:22)
DD24   TCCTCCCTTACGGGTTAGAA   (SEQ ID No:24)
25 B. hermsii ATATACCTTCCTCCCTTTCGGG   (SEQ ID No:4
                                              antisense)

In addition, given the information provided
in FIG. 6 regarding the B. hermsii 16S rRNA gene
30 sequence, probes specific for the detection of this
Borrelia species may be designed. Such probes include,
for example, sequences which incorporate specific base
substitutions between B. burgdorferi and B. hermsii.
Illustrative probes include:

35

DD25 (5'3') AAAGCGACAGAACAGTGA   (SEQ ID No: 29)
      and the complementary sequence thereto

DD27 (5'-3') GGACTCAGATAAGACTGC   (SEQ ID No: 30)
40   and the complementary sequence thereto

-36-

Example 6. Nested Systems

The nested primer system may be performed in a single tube eliminating the carry over contamination which can be a problem with conventional nested systems. In the single tube coamplification PCR, the external primers DD17 3'-GTCACCTC CTCACTAAACATACCT-5' (SEQ ID No: 17) and DD18 5'-GTCCAGCATCTGTTACCAGCATGTAAT-3' (SEQ ID No: 18) have melting temperatures of 66°C and 70°C, respectively, and the internal primers DD19 3'-ATACCTTCCTCCCTTA-5' (SEQ ID No: 19) and DD20 5'-GTGCTACAATGGCCT-3' (SEQ ID No: 20) have melting temperatures of 48°C and 52°C, respectively.

A "Mastermix" composed of 20 ul 50% glycerol (10% total), 12.5 ul glass distilled H_2O, 10.0 ul 10X buffer (GeneAmp), 2.5 ul each dNTP (250 uM total), 1 ul DD17 (SEQ ID No: 17) (5 uM), 1 ul DD18 (SEQ ID No: 18) (5 uM), 1 ul DD19 (SEQ ID No: 19) (50 uM), 1 ul DD20 (SEQ ID No: 20) (50 uM) and 1 ul Taq polymerase (5 units/100 ul reaction) were added to a 50 ul sample of human urine (prepared as described in Example 11).

The solution was heated first to 95°C for 5 minutes followed by 25 cycles of a denaturing step at 95°C for 45 seconds and an annealing step at 60°C for 45 seconds. For the next 25 cycles, the denaturing step was performed at 95°C for 45 seconds and an annealing step at 45°C for 45 seconds. A final extension step was performed at 72°C for 10 minutes, followed by a 10°C soak in the thermocycler overnight.

As controls, 50 ul of sample were added to a 50 ul volume of the Mastermix containing either the set of external or internal primers. In the PCR using the external primers DD17 (SEQ ID No: 17) and DD18 (SEQ ID No: 18), the cycling conditions were identical except that the entire 50 cycles were run using an anneal

temperature of 60°C. Similarly, in the PCR using the internal primers DD19 (SEQ ID No: 19)and DD20 (SEQ ID No: 20), the entire 50 cycles were run using an anneal temperature of 45°C.

FIG. 5 provides the results of both the control primer amplification products and the coamplification products using both sets of primers. The legend to the lane samples is as follows:

Lane No.	DNA/Control	Concentration
2, 17, 33	B. burgdorferi 210	2000 fg
3, 18, 34	B. burgdorferi 210	200 fg
4, 19, 35	B. burgdorferi 210	20 fg
5, 20, 36	B. burgdorferi 210	2 fg
6, 21, 37	B. burgdorferi 211	10^4 copies
7, 22, 38	B. burgdorferi BPH11 (DN 1-2-7)	10^4 copies
8, 23, 39	B. hermsii 209	10^3 copies
9, 24, 40	B. coriaceae	1.5×10^3 copies
10, 25, 41	B. parkeri	1.5×10^3 fg
11, 26, 42	B. turicatae	1.5×10^3 fg
12, 27, 43	B. anserina	1.5×10^3 fg

1, 16, 31 and 48 are molecular weight markers of ϕX174 cut with Hae III (New England Biolabs) 13-15, 28-30 and 44-46 are negative controls 32 and 47 are blank

The sensitivity for the external "stand alone" primary amplification system (primers DD17 (SEQ ID No: 17) and DD18 (SEQ ID No: 18)) shown in Lanes 1-15 for B. burgdorferi is at 200 fg (Lane 3). Both European (211) and Californian (BPH11 - DN 1-2-7) strains are amplified by these primers (Lanes 6 and 7). B. hermsii and B. coriaceae are weakly amplified (Lanes 8 and 9). B. parkeri, B. turicatae and B. anserina all amplify well with these primers (Lanes 10-12).

The sensitivity for the internal "stand alone" primary amplification system (primers DD19 (SEQ ID No: 19)and DD20 (SEQ ID No: 20)) shown in Lanes

17-30 for B. burgdorferi DNA is at approximately 1
copy, 2 fg (Lanes 17-20). The primers amplify European
(211 - Lane 21) and Californian (BPH11 - DN 1-2-7 -
Lane 22) strains well. B. hermsii and B. coriaceae

5   show bands with moderate intensity (Lanes 23 and 24).
B. parkeri and B. turicatae amplify well with these
primers (Lanes 25 and 26). B. anserina was not
amplified with these internal primers during primary
amplification (Lane 27).

10   In the coamplification reaction using both
sets of primers, the nested product (DD19/DD20) was
produced for all Borrelia species tested. When the
initial sample concentration was 10^3 fg or lower, the
nested product was preferentially produced over

15   external product (Lanes 33-35, 39-43). When the
initial sample concentration was above 10^3 fg, more
external product was produced whereas the internal,
nested product was produced less efficiently (Lanes 37
and 38).

20

Example 7. Amplification of and Sequencing of B. hermsii
       The primers DD02 (SEQ ID No: 2) and DD06 (SEQ
ID No: 6) were used to amplify the 16S ribosomal RNA
gene from B. hermsii and B. burgdorferi. The amplified

25   products were subcloned into sequencing vectors as
described in Maniatis Vol. 1 at Section 1.53.
       From this reaction approximately 366
nucleotides of B. hermsii and B. burgdorferi were
sequenced using the Sequenase kit and the instructions

30   provided therein. The sequencing results are shown in
FIG. 6. The B. hermsii sequence is shown in the middle
line. The corresponding B. burgdorferi sequence, shown
in the top line of FIG. 6, was aligned for maximum
identity. As depicted in the bottom line of this

35   figure, mismatches between the two species are shown.

-39-

These regions of mismatch are advantageous for probe design.

Example 8. Sample Preparation for Direct Specimen Assay
5   For tick preparations, whole ticks (or the tick midgut) were crushed with sterile Pasteur pipets and suspended in 100 ul Tris buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0). The solution was boiled at 100 °C for 15 min, spun in a microfuge for 5 min, and then
10   the crude lysate was removed with a positive displacement pipet. Approximately 50 ml of the lysate is placed directly into the PCR reaction for amplification of the organism from the insect material.

15   Example 9. Preparation of Clinical Samples
          The following protocols may be used to prepare DNA or RNA from blood cells or from tissue. To prepare the sample from blood cells, the procedure described by Kawasaki (see "Sample Preparation from
20   Blood, Cells and Other Fluids" in PCR Protocols, A Guide to Methods and Applications, eds. Innis et al., Academic Press, 1990) is employed. Briefly, approximately 50 ul of whole blood from a patient suspected of having Lyme disease is mixed with 0.5 ml
25   of TE in a 1.5 ml microfuge tube, which is then spun for 10 sec at 13,000 x g. The pellet is resuspended in 0.5 ml of TE by vortexing and pelleted as before. This procedure is repeated twice more and the final pellet is resuspended in 100 ul of K buffer (PCR buffer
30   without gelatin or BSA, containing 1% Laureth 12 or 0.5% Tween 20 and 100 ug/ml of fresh Proteinase K) and incubated as above. Approximately 10 ul of this solution is used in a PCR.
          From a tissue sample, the tissue must first
35   be homogenized employing standard procedures, and the

-40-

homogenate is subjected to standard DNA isolation procedures (see for example, Maniatis et al., supra) to purify the DNA. The purified DNA is then used in a PCR.

5   Example 10.   Detection of Target DNA in Cerebrospinal Fluid

       Ten ul (direct addition) cerebrospinal fluid (CSF) added to 40 ul water and 50 ul Mastermix, were tested in a PCR with primers DD02 (SEQ ID No: 2) and
10   DD06 (SEQ ID No: 6). The solution was heated first to 95°C for 5 minutes followed by 50 cycles of a denaturing step at 95°C for 25 seconds and an annealing step at 55°C for 25 seconds. A final extension step was performed at 72°C for 10 minutes, followed by a
15   10°C soak overnight in the thermocycler.

       The amplified DNA was immobolized on a nylon membrane and treated under prehybridizing conditions. About 10 pmoles of kinased probe DD04 (SEQ ID No: 4) was added to the membrane in solution and hybridization
20   was run for 3-5 hours at 60°C. The membrane was rinsed in 5x SSPE, 0.1% SDS at room temperature, washed in 2x SSPE, 0.1% SDS at 60°C for 30 minutes followed by a wash in 0.2x SSPE, 0.1% SDS at 60°C for 10 minutes. The blots were exposed to X-ray film and developed.

25   Out of nineteen patients tested, positive results were observed in nine patients who had been previously diagnosed with neuroborreliosis.

       Example 11. Detection of Target DNA in Synovial Fluid
30   Two methods for sample preparation of synovial fluid have been evaluated. In the first method, 200 ul of synovial fluid were boiled at 100°C for 5 minutes and the sample was centrifuged (16,000 x g) for 30 minutes at 4°C. Approximately 10 ul of the
35   supernatant were used in a 100 ul PCR reaction using

-41-

primers DD02 (SEQ ID No: 2) and DD06 (SEQ ID No: 6)
followed by hybridization with probe DD04 (SEQ ID No:
4).

In the second method, 200 ul of synovial
fluid was extracted with phenol:
chloroform:isoamylalcohol (25:24:1) and the DNA
precipitated with ethanol. About 10 ul of the
extracted sample DNA was resuspended in 30 ul Tris-HCl,
EDTA and used in a 100 ul PCR reaction with the same
primers and probe identified above.

In this study, six patients were tested;
three were diagnosed with Lyme arthritis, the other
patients had different types of arthritis such
osteoarthritis or rheumatoid arthritis. Positive
results were observed using either sample preparation
method in the three patients diagnosed with Lyme
arthritis.

Example 12. Sample Preparation from Urine.

About 1 ml of urine was added to a 1.5 ml
microfuge tube and centrifuged (16,000 x g) for 5
minutes at room temperature. The supernatant was
removed and 250 ul sterile TE buffer was added to
resuspend the pellet. The resuspended material was
boiled at 100°C for 10 minutes. The material was
refrigerated at 4°C until ready for use in PCR.

About 50 ul of the lysate was used in a PCR
reaction employing the primers DD02 (SEQ ID No: 2) and
DD06 (SEQ. ID No: 6), followed by detection with the
probe DD04 (SEQ ID No: 4) as described for the CSF
detection example. Using the "primary system" (50
cycles amplification followed by dot blot detection)
for 36 samples, positive results were observed for a
patient with early Lyme disease who had the B.
burgdorferi specific ECM lesion (erythema chronicum

-42-

migrans).   A   positive   result   was   observed   in   20
patients tested with early disease using the nested
primer system (see Example 6 for conditions).
                                        SEQUENCE LISTING

(1) GENERAL INFORMATION:

    (i) APPLICANT: White Ph.D, Thomas J.
                   Dodge, Deborah E.

    (ii) TITLE OF INVENTION: Method for Diagnosis of Lyme Disease

    (iii) NUMBER OF SEQUENCES: 29

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        (F) ZIP: 94608

    (v) COMPUTER READABLE FORM:
        (A) MEDIUM TYPE: Floppy disk
        (B) COMPUTER: IBM PC compatible
        (C) OPERATING SYSTEM: PC-DOS/MS-DOS
        (D) SOFTWARE: PatentIn Release #1.0, Version #1.25

    (vi) CURRENT APPLICATION DATA:
        (A) APPLICATION NUMBER:
        (B) FILING DATE:
        (C) CLASSIFICATION:

    (vii) PRIOR APPLICATION DATA:
        (A) APPLICATION NUMBER: US 489,676
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        (C) TELEX: 4992659

(2) INFORMATION FOR SEQ ID NO:1:
    (i) SEQUENCE CHARACTERISTICS:

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                    (A) LENGTH: 505 base pairs
                    (B) TYPE: nucleic acid
                    (C) STRANDEDNESS: single
                    (D) TOPOLOGY: linear

          (ii) MOLECULE TYPE: DNA (genomic)

          (iii) HYPOTHETICAL: NO

          (iv) ANTI-SENSE: NO

          (ix) FEATURE:
                    (A) NAME/KEY: unsure
                    (B) LOCATION: complement (35..36)

          (ix) FEATURE:
                    (A) NAME/KEY: unsure
                    (B) LOCATION: complement (41)

          (ix) FEATURE:
                    (A) NAME/KEY: unsure
                    (B) LOCATION: complement (67)

          (ix) FEATURE:
                    (A) NAME/KEY: unsure
                    (B) LOCATION: complement (164..165)

          (ix) FEATURE:
                    (A) NAME/KEY: unsure
                    (B) LOCATION: complement (167)

          (ix) FEATURE:
                    (A) NAME/KEY: unsure
                    (B) LOCATION: complement (169)


          (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GTATAGGAAG ATAGTTAGAG ATAATTATTC CCCGNNTGGG NCTATATACA GGTGCTGCAT   60

GGTTGTNGTC AGCTCGTGCT GTGAGGTGTT GGGTTAAGTC CCGAACGAGG AACCCTTGTT  120

ATCTGTTACC AGCATGTAAT GGTGGGGACT CAGATAAGAC TGCNNGNTNG ATAAGTCGGA  180

GGAAGGTGAG GATGACGTCA AATCATCATG GCCCTTATGT CCTGGGCTAC ACACGTGCTA  240

CAATGGCCTG TACAAAGCGA CAGAACAGTG ATGTGAAGCA AAAACGCATA AAGCAGGTCT  300

CAGTCCGGAT TGAAGTCTGA AACTCGACTT CATGAAGTTG GAATCGCTAG TAATCGTATA  360

TCAGAATGAT ACGGTGAATA CGTTCTCGGG CCTTGTACAC ACCGCCCGTC ACACCACCCG  420
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−44−

AGTTGAGGAT ACCCGAAGCT ATTATTCTAA CCCGTAAGGG AGGAAGGTAT TTAAGGTATG 480

TTTAGTGAGG GGGGTGAAGG TATTT   505

```
5        (2) INFORMATION FOR SEQ ID NO:2:
              (i) SEQUENCE CHARACTERISTICS:.
                   (A) LENGTH: 18 base pairs
                   (B) TYPE: nucleic acid
                   (C) STRANDEDNESS: single
10                 (D) TOPOLOGY: linear

              (ii) MOLECULE TYPE: DNA (genomic)

              (iii) HYPOTHETICAL: NO
15
              (iv) ANTI-SENSE: NO

              (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

20       TCCATACAAA TCACTCCC                                        18


         (2) INFORMATION FOR SEQ ID NO:3:
              (i) SEQUENCE CHARACTERISTICS:
25                 (A) LENGTH: 19 base pairs
                   (B) TYPE: nucleic acid
                   (C) STRANDEDNESS: single
                   (D) TOPOLOGY: linear

30            (ii) MOLECULE TYPE: DNA (genomic)

              (iii) HYPOTHETICAL: NO

              (iv) ANTI-SENSE: NO
35
              (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

         CACCCGAGTT GAGGATACC                                       19
40
         (2) INFORMATION FOR SEQ ID NO:4:
              (i) SEQUENCE CHARACTERISTICS:
                   (A) LENGTH: 21 base pairs
                   (B) TYPE: nucleic acid
45                 (C) STRANDEDNESS: single
                   (D) TOPOLOGY: linear

              (ii) MOLECULE TYPE: DNA (genomic)

50            (iii) HYPOTHETICAL: NO

              (iv) ANTI-SENSE: NO
```

```
        (ix) FEATURE
                (A) NAME/KEY: misc_difference
                (B) LOCATION: replace(5, "c")
                (D) OTHER INFORMATION: /standard_name= "base
                                substitution" /note= "At position
                                5 the base may be either T or C"

        (ix) FEATURE
                (A) NAME/KEY: misc_difference
                (B) LOCATION: replace(21, "c")
                (D) OTHER INFORMATION: /standard_name= "base
                                substitution" /note= "At position
                                21 the base may be T, C or absent"

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

    CCCGTAAGGG AGGAAGGTAT T                                      20


    (2) INFORMATION FOR SEQ ID NO:5:
        (i) SEQUENCE CHARACTERISTICS:
                (A) LENGTH: 20 base pairs
                (B) TYPE: nucleic acid
                (C) STRANDEDNESS: single
                (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: DNA (genomic)

        (iii) HYPOTHETICAL: NO

        (iv) ANTI-SENSE: NO

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

    ATAGTTAGAG ATAATTATTC                                        20


    (2) INFORMATION FOR SEQ ID NO:6:
        (i) SEQUENCE CHARACTERISTICS:
                (A) LENGTH: 20 base pairs
                (B) TYPE: nucleic acid
                (C) STRANDEDNESS: single
                (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: DNA (genomic)

        (iii) HYPOTHETICAL: NO

        (iv) ANTI-SENSE: NO

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

    ATCTGTTACC AGCATGTAAT                                        20
```

(2) INFORMATION FOR SEQ ID NO:7:
    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 17 base pairs
        (B) TYPE: nucleic acid
5   (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

10 (iii) HYPOTHETICAL: NO

    (iv) ANTI-SENSE: NO

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:
15
CGTGCTACAA TGGCCTG   17

(2) INFORMATION FOR SEQ ID NO:8:
20 (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 25 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear
25
    (ii) MOLECULE TYPE: DNA (genomic)

    (iii) HYPOTHETICAL: NO

30 (iv) ANTI-SENSE: NO

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GGAAGATAGT TAGAGATAAT TATTC   25
35

(2) INFORMATION FOR SEQ ID NO:9:
    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 26 base pairs
40   (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)
45
    (iii) HYPOTHETICAL: NO

    (iv) ANTI-SENSE: NO

50 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CTTGTTATCT GTTACCAGCA TGTAAT   26

−47−

(2) INFORMATION FOR SEQ ID NO:10:
        (i) SEQUENCE CHARACTERISTICS:
                (A) LENGTH: 21 base pairs
                (B) TYPE: nucleic acid
                (C) STRANDEDNESS: single
                (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: DNA (genomic)

        (iii) HYPOTHETICAL: NO

        (iv) ANTI-SENSE: NO

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

TATCTGTTAC CAGCATGTAA T   21

(2) INFORMATION FOR SEQ ID NO:11:
        (i) SEQUENCE CHARACTERISTICS:
                (A) LENGTH: 15 base pairs
                (B) TYPE: nucleic acid
                (C) STRANDEDNESS: single
                (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: DNA (genomic)

        (iii) HYPOTHETICAL: NO

        (iv) ANTI-SENSE: NO

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

ACACACGTGC TACAA   15

(2) INFORMATION FOR SEQ ID NO:12:
        (i) SEQUENCE CHARACTERISTICS:
                (A) LENGTH: 25 base pairs
                (B) TYPE: nucleic acid
                (C) STRANDEDNESS: single
                (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: DNA (genomic)

        (iii) HYPOTHETICAL: NO

        (iv) ANTI-SENSE: NO

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

-48-

CAGATAAGAC TGCCGGTGAT AAGTC   25

(2) INFORMATION FOR SEQ ID NO:13:
       (i) SEQUENCE CHARACTERISTICS:
           (A) LENGTH: 881 base pairs
           (B) TYPE: nucleic acid
           (C) STRANDEDNESS: single
           (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (iii) HYPOTHETICAL: NO

    (iv) ANTI-SENSE: NO

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

TAGAGGGGGG TAGAATTCCA GGTGTAGCGG TGAAATGCGT AGAGATCTGG AGGAATACCG 60

GTGGCGAAGG CGGCCCCCTG GACGAAGACT GACGCTCAGG TGCGAAAGCG TGGGGAGCAA 120

ACAGGATTAG ATACCCTGGT AGTCCACGCC GTAAACGATG TCGACTTGGA GGTTGTGCCC 180

TTGAGGCGTG GCTTCCGGAG CTAACGCGTT AAGTCGACCG CCTGGGGAGT ACGGCCGCAA 240

GGTTAAAACT CAAATGAATT GACGGGGGCC CGCACAAGCG GTGGAGCATG TGGTTTAATT 300

CGATGCAACG CGAAGAACCT TACCTGGTCT TGACATCCAC GGAAGTTTTC AGAGATGAGA 360

ATGTGCCTTC GGGAACCGTG AGACAGGTGC TGCATGGCTG TCGTCAGCTC GTGTTGTGAA 420

ATGTTGGGTT AAGTCCCGCA ACGAGCGCAA CCCTTATCCT TTGTTGCCAG CGGTCCGGCC 480

GGGAACTCAA AGGAGACTGC CAGTGATAAA CTGGAGGAAG GTGGGGATGA CGTCAAGTCA 540

TCATGGCCCT TACGACCAGG GCTACACACG TGCTACAATG GCGCATACAA AGAGAAGCGA 600

CCTCGCGAGA GCAAGCGGAC CTCATAAAGT GCGTCGTAGT CCGGATTGGA GTCTGCAACT 660

CGACTCCATG AAGTCGGAAT CGCTAGTAAT CGTGGATCAG AATGCCACGG TGAATACGTT 720

CCCGGGCCTT GTACACACCG CCCGTCACAC CATGGGAGTG GGTTGCAAAA GAAGTAGGTA 780

GCTTAACCTT CGGGAGGGCG CTTACCACTT TGTGATTCAT GACTGGGGTG AAGTCGTAAC 840

AAGGTAACCG TAGGGGAACC TGCGGTTGGA TCACCTCCTT A   881

(2) INFORMATION FOR SEQ ID NO:14:
       (i) SEQUENCE CHARACTERISTICS:
           (A) LENGTH: 20 base pairs

-49-

```
                          (B) TYPE: nucleic acid
                          (C) STRANDEDNESS: single
                          (D) TOPOLOGY: linear

  5             (ii) MOLECULE TYPE: DNA (genomic)

                (iii) HYPOTHETICAL: NO

                (iv) ANTI-SENSE: YES

 10             (xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

                GGGCATTCCC TCCTTCCATA                                    20

 15     (2) INFORMATION FOR SEQ ID NO:15:
            (i) SEQUENCE CHARACTERISTICS:
                  (A) LENGTH: 17 base pairs
                  (B) TYPE: nucleic acid
 20               (C) STRANDEDNESS: single
                  (D) TOPOLOGY: linear

                (ii) MOLECULE TYPE: DNA (genomic)

 25             (iii) HYPOTHETICAL: NO

                (iv) ANTI-SENSE: NO

                (xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:
 30
        TAACCCGTAA GGGAGGA                                               17


        (2) INFORMATION FOR SEQ ID NO:16:
 35         (i) SEQUENCE CHARACTERISTICS:
                  (A) LENGTH: 17 base pairs
                  (B) TYPE: nucleic acid
                  (C) STRANDEDNESS: single
                  (D) TOPOLOGY: linear
 40
                (ii) MOLECULE TYPE: DNA (genomic)

                (iii) HYPOTHETICAL: NO

 45             (iv) ANTI-SENSE: YES

                (xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

        ATTGGGCATT CCCTCCT                                              17
 50
```

(2) INFORMATION FOR SEQ ID NO:17:
    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 25 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (iii) HYPOTHETICAL: NO

    (iv) ANTI-SENSE: NO

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

TCCATACAAA TCACTCCGTC CACTG   25

(2) INFORMATION FOR SEQ ID NO:18:
    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 27 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (iii) HYPOTHETICAL: NO

    (iv) ANTI-SENSE: NO

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

GTCCAGCATC TGTTACCAGC ATGTAAT   27

(2) INFORMATION FOR SEQ ID NO:19:
    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 16 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (iii) HYPOTHETICAL: NO

    (iv) ANTI-SENSE: NO

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

ATTCCCTCCT TCCATA   16

(2) INFORMATION FOR SEQ ID NO:20:
        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 15 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
                (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: DNA (genomic)

        (iii) HYPOTHETICAL: NO

        (iv) ANTI-SENSE: NO

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

GTGCTACAAT GGCCT   15

(2) INFORMATION FOR SEQ ID NO:21:
        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 27 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: DNA (genomic)

        (iii) HYPOTHETICAL: NO

        (iv) ANTI-SENSE: NO

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

AACCCGTAAG GGAGGAAGGT ATTTAAG   27

(2) INFORMATION FOR SEQ ID NO:22:
        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 27 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: DNA (genomic)

        (iii) HYPOTHETICAL: NO

        (iv) ANTI-SENSE: YES

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

TTGGGCATTC CCTCCTTCCA TAAATTC   27

(2) INFORMATION FOR SEQ ID NO:23:
        (i) SEQUENCE CHARACTERISTICS:
                (A) LENGTH: 20 base pairs
                (B) TYPE: nucleic acid
                (C) STRANDEDNESS: single
                (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: DNA (genomic)

        (iii) HYPOTHETICAL: NO

        (iv) ANTI-SENSE: NO

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

TTCTAACCCG TAAGGGAGGA   20

(2) INFORMATION FOR SEQ ID NO:24:
        (i) SEQUENCE CHARACTERISTICS:
                (A) LENGTH: 20 base pairs
                (B) TYPE: nucleic acid
                (C) STRANDEDNESS: single
                (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: DNA (genomic)

        (iii) HYPOTHETICAL: NO

        (iv) ANTI-SENSE: YES

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

AAGATTGGGC ATTCCCTCCT   20

(2) INFORMATION FOR SEQ ID NO:25:
        (i) SEQUENCE CHARACTERISTICS:
                (A) LENGTH: 20 base pairs
                (B) TYPE: nucleic acid
                (C) STRANDEDNESS: single
                (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: DNA (genomic)

        (iii) HYPOTHETICAL: NO

        (iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

ACAAGCGGTG GAGCATGTGG   20

5

(2) INFORMATION FOR SEQ ID NO:26:
         (i) SEQUENCE CHARACTERISTICS:
                 (A) LENGTH: 22 base pairs
                 (B) TYPE: nucleic acid
10   (C) STRANDEDNESS: single
                 (D) TOPOLOGY: linear

         (ii) MOLECULE TYPE: DNA (genomic)

15   (iii) HYPOTHETICAL: NO

         (iv) ANTI-SENSE: NO

         (xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:
20
GGTTAAGTCC CGCAACGAGC GC   22

(2) INFORMATION FOR SEQ ID NO:27:
25   (i) SEQUENCE CHARACTERISTICS:
                 (A) LENGTH: 20 base pairs
                 (B) TYPE: nucleic acid
                 (C) STRANDEDNESS: single
                 (D) TOPOLOGY: linear
30
         (ii) MOLECULE TYPE: DNA (genomic)

         (iii) HYPOTHETICAL: NO

35   (iv) ANTI-SENSE: NO

         (xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

TACGTTCCCG GGCCTTGTAC   20
40

(2) INFORMATION FOR SEQ ID NO:28:
         (i) SEQUENCE CHARACTERISTICS:
                 (A) LENGTH: 20 base pairs
45   (B) TYPE: nucleic acid
                 (C) STRANDEDNESS: single
                 (D) TOPOLOGY: linear

         (ii) MOLECULE TYPE: DNA (genomic)
50
         (iii) HYPOTHETICAL: NO

-54-

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

5   AGGAGGTGAT CCAACCGACA   20

(2) INFORMATION FOR SEQ ID NO:29:
          (i) SEQUENCE CHARACTERISTICS:
10   (A) LENGTH: 18 base pairs
                  (B) TYPE: nucleic acid
                  (C) STRANDEDNESS: single
                  (D) TOPOLOGY: linear

15   (ii) MOLECULE TYPE: DNA (genomic)

          (iii) HYPOTHETICAL: No

          (iv) ANTI-SENSE: No
20
          (xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

        AAAGCGACAG AACAGTGA   18

25
2) INFORMATION FOR SEQ ID NO:30:
          (i) SEQUENCE CHARACTERISTICS:
                  (A) LENGTH: 18 base pairs
                  (B) TYPE: nucleic acid
30   (C) STRANDEDNESS: single
                  (D) TOPOLOGY: linear

          (ii) MOLECULE TYPE: DNA (genomic)

35   (iii) HYPOTHETICAL: No

          (iv) ANTI-SENSE: No

          (xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:
40
        GGACTCAGAT AAGACTGC   18

            Although the foregoing invention has been
        described in some detail for the purpose of
45   illustration, it will be obvious that changes and
        modifications may be practiced within the scope of the
        appended claims by those of ordinary skill in the art.

-55-

We claim:

1. A method for determining the presence of a _Borrelia_ polynucleotide in a sample suspected of containing said _Borrelia_ polynucleotide, wherein said _Borrelia_ polynucleotide comprises a selected target region, said method comprising:

(a) amplifying the target region to a detectable level, wherein said target region contains a _Borrelia_ 16S ribosomal polynucleotide sequence;

(b) providing to the product of step (a) a polynucleotide probe capable of hybridizing to the _Borrelia_ 16S ribosomal polynucleotide sequence in the target region;

(c) incubating the amplified target region with the polynucleotide probe under conditions which allow specificity of hybrid duplexes; and

(d) detecting hybrids formed between the amplified target region and the polynucleotide probe.

2. The method of claim 1, wherein a pair of DNA primers are used to amplify the target region, said primers comprising a nucleic acid sequence which is substantially complementary to a substantially conserved region among the nucleic acid sequence of the 16S ribosomal DNA of _Borrelia_ to hybridize therewith and act as a point of initiation of synthesis in an amplification reaction.

3. The method of claim 2, wherein each primer is between 15 and 35 nucleotides in length.

4.   The method of claim 1, wherein the probe comprises a region derived from DNA sufficiently complementary to the 16S ribosomal RNA gene of <u>Borrelia</u> <u>burgdorferi</u> to hybridize therewith allowing for detection of the target region.

5.   The method of claim 4, wherein the probe comprises a sequence of at least 15 nucleotides located from about nucleotide 934 through about 1539 of the <u>Borrelia</u> nucleic acid sequence shown in Figure 1 (SEQ ID No: 1), or a sequence complementary thereto.

6.   The method of claim 5, wherein the probe comprises a sequence of at least 15 nucleotides located from about nucleotide 1121 through about 1489 shown in Figure 1 (SEQ ID No: 1).

7.   The method of claim 6, wherein the sequence is 3'-CCCGNAAGGGAGGAAGGTATN-5', wherein N at position 5 is C or T and N at position 21 is T, C or zero (SEQ ID No: 4).

8.   The method of claim 7, wherein the sequence is 5'-CCCGTAAGGGAGGAAGGTAT-3'.

9.   The method of claim 1 wherein the step (c) incubations are performed using stringent hybridization and wash conditions, allowing for detection of <u>Borrelia</u> <u>burgdorferi</u>.

10.   The method of claim 1 wherein the step (c) incubations are performed using low stringency hybridization and wash conditions, allowing for detection of <u>Borrelia</u> <u>hermsii</u>.

-57-

11.   An oligonucleotide probe for detecting
or monitoring Borrelia species nucleic acid in a
sample, comprising a sequence capable of hybridizing to
amplified 16S ribosomal DNA of Borrelia, or a sequence
5   complementary thereto.

12.   The oligonucleotide probe of claim 11,
wherein the DNA of the amplified 16S ribosomal RNA is
from Borrelia burgdorferi.
10

13.   The oligonucleotide probe of claim 11,
wherein the sequence comprises at least 12 nucleotides
located from about nucleotide 934 through about 1539
shown in Figure 1 (SEQ ID No: 1).
15

14.   The oligonucleotide probe of claim 11,
wherein the sequence comprises at least 15 nucleotides
located from about nucleotide 1121 through about 1489
shown in Figure 1 (SEQ ID No: 1).
20

15.   The oligonucleotide probe of claim 14,
wherein the sequence is 5'-CCCGNAAGGGAGGAAGGTATN-3'
wherein N at position 5 is C or T and N at position 21
is T, C or zero (SEQ ID No: 4).
25

16.   The oligonucleotide probe of claim 11,
which further comprises a label.

17.   A method for amplifying a target region
30   in a 16S ribosomal Borrelia polynucleotide comprising:
         (a) providing a set of primers, wherein
a first primer contains a region which is sufficiently
complementary to a sequence in a sense strand of 16S
ribosomal Borrelia polynucleotide to prime the
35   synthesis of a DNA strand which is a complement to the

-58-

sense strand of the target region, and a second primer which contains a region which is sufficiently complementary to a sequence in an antisense strand of 16S ribosomal Borrelia polynucleotide to prime the
5   synthesis of a DNA strand which is a complement to the antisense strand of the target region;

(b) providing a sample suspected of containing the target region;

(c) contacting the sample with four
10   different deoxynucleoside triphosphates, the first and second primers, and a primer- and template-dependent polymerizing agent, under conditions whereby the primers are extended using the target region as template, yielding primer extension products;
15   (d) treating the primer extension products under denaturing conditions to separate the primer extension products from their templates; and

(e) repeating steps (c) and (d) until the target sequence is amplified to a level which
20   allows it to be detected above non-target levels in the sample.

18.   The method of claim 17, wherein the sample is a biological sample.
25

19.   The method of claim 18, wherein the sample is a mammalian biological sample.

20.   The method of claim 19, wherein the
30   mammalian biological sample is human.

21.   The method of claim 17, wherein the Borrelia species is B. burgdorferi or B. hermsii.

22. The method of claim 17, wherein at least one additional primer, which is different from the first or second primer, is added in step (e) which provides an amplified product that is smaller than and contained within the sequence being amplified in step (c).

23. The method of claim 22, wherein a second set of primers are employed in step (e), whereby said primers are different from the primers employed in step (a) and provide for the amplification of a smaller sequence contained within the sequence being amplified in step (c).

24. The method of claim 17, wherein each primer comprises a nucleic acid sequence which is substantially complementary to a substantially conserved region among the nucleic acid sequence of the 16S ribosomal polynucleotide of Borrelia to hybridize therewith and act as a point of initiation of synthesis in an amplification reaction.

25. The method of claim 24, wherein each primer comprises at least 15 nucleotides located from about nucleotide 934 through about 1489 shown in Figure 1 (SEQ ID No: 1).

26. The method of claim 25, wherein each primer comprises at least 15 nucleotides located from about nucleotide 1121 through about 1489 shown in Figure 1.

27. The method of claim 26, wherein one of the primers contains the sequence: 5'-ATCTGTTACCAGCATGTAAT-3' (SEQ ID No: 6).

-60-

28. The method of claim 26, wherein one of the primers contains the sequence: 3'-CCCTCACTAAACATACCT-5' (SEQ ID No: 2).

29. The method of claim 17, wherein one primer contains the sequence: 5'-ATCTGTTACCAGCATGTAAT-3' (SEQ ID No: 6) and the other primer contains the sequence: 3'-CCCTCACTAAACATACCT-5' (SEQ ID No: 2).

30. The method of claim 17 wherein an amount of glycerol effective to increase amplification is added during the extension step of (c).

31. The method of claim 30 wherein the amount of glycerol is 5%-20% (v/v).

32. The method of claim 17, further comprising the steps of:
(e) providing a polynucleotide probe containing a sequence which is sufficiently complementary to hybridize to a unique sequence in the target region;
(f) incubating the amplified nucleic acid with the polynucleotide probe under conditions which allow specificity of hybrid duplexes; and
(g) detecting hybrids formed between the amplified target region and the polynucleotide probe.

33. The method of claim 17 further comprising a sequence which, when hybridized with its complement, encodes a restriction enzyme site.

-61-

34. The method of claim 32, wherein one of the primers contains the sequence: 5'-ATCTGTTACCAGCATGTAAT-3' (SEQ ID No: 6) and the other primer contains the sequence: 3'-CCCTCACTAAACATACCT-5' (SEQ ID No: 2).

35. The method of claim 32 wherein the sequence of the nucleotide probe is 5'-CCCGTAAGGGAGGAAGGTAT-3' (SEQ ID No: 4) or its complementary sequence.

36. The method of claim 32 wherein the sequence of the nucleotide probe is 5'-AAAGCGACAGAACAGTGA-3' (SEQ ID No: 30) or its comlementary sequence.

37. A polymerase chain reaction (PCR) kit for amplifying a target region in a Borrelia 16S ribosomal polynucleotide, comprising:

(a) a first container in which are stored a set of primers, one for each strand of the Borrelia nucleic acid sequence to be amplified, which primers are sufficiently complementary to substantially conserved regions among the Borrelia 16S ribosomal DNA to hybridize therewith, such that an extension product synthesized from each primer is substantially complementary to a strand of the Borrelia DNA sequence being amplified, such that the extension product synthesized from each primer, when separated from its complement, can serve as a template for the synthesis of the extension product of the other primer; and

(b) one or more additional containers in which are stored reagents for performing PCR amplification and, optionally, probe hybridization.

-62-

38.   The kit of claim 37, wherein said
primers comprise a nucleic acid sequence which sequence
is   sufficiently   complementary   to   substantially
conserved regions among the nucleic acid of the 16S
5   ribosomal DNA of Borrelia to hybridize therewith and
act as a point of initiation of synthesis in an
amplification   reaction,   wherein   said   substantially
conserved region is at least 12 nucleotides long.

10   39.   The kit of claim 38, wherein said
primers are between 15 and 35 nucleotides in length.

40.   The PCR kit of claim 37, wherein each
primer comprises at least 15 nucleotides located from
15   about nucleotide 1121 through about 1489 shown in
Figure 1 (SEQ ID No: 1).

41.   The PCR kit of claim 40, wherein one of
the   primers   contains   the   sequence:
20   5'-ATCTGTTACCAGCATGTAAT-3' (SEQ ID No: 6) and the other
primer contains the sequence: 3'-CCCTCACTAAACATACCT-5'
(SEQ ID No: 2).

42.   The PCR kit of claim 37, wherein said
25   stored   reagents   include   dNTPs   and   a   primer-   and
template-dependent polynucleotide polymerizing agent.

43.   The PCR kit of claim 42, wherein said
polynucleotide polymerizing agent is a thermostable DNA
30   polymerase.

FIG. 1A

```
Seq 2: TAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCG   720

Seq 1:
Seq 2: GTGGCGAAGGCGGCCCCCTGGACGAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAA   780

Seq 1:
Seq 2: ACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTTGGAGGTTGTGCCC   840

Seq 1:
Seq 2: TTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAA   900

Seq 1:
Seq 2: GGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATT   960

                         *****   *  *  **   *                 *
                             GTATA  GGANGATAGTT  AGAGATA
Seq 1:
Seq 2: CGATGCAACGCGAAGAACCTTACCTGGTCTTGACATCCAC GGAAGTT  TTCAGAGATC   1017
```

FIG. 1B

```
            *   *    *     *       *   *    *  *                        *
Seq 1: ATTAT TCCCCGNNTGGGN CTAT ATACAGGTGCTGCTGCATGGTTGTTGTNGTCAGCTCGTGCT
Seq 2: AGAATGTGCCT TC GGGAACCGTGAGACAGGTGCTGCTGCATGGCTGTGTCGTCGTCAGCTCGTGTT      1075
                                                              DD06 →

       **        *    *      *          *        *   *       *
Seq 1: GTGAGGTGTTGGGTTAAGTCCCG AACGAG G AACCCTTGTTATCTGTTACCAGCATGT
Seq 2: GTGAAATGTTGGGTTAAGTCCCGCAACCCTTATCCTTGTTGCCAGCG GT      1134

       **      **    *    *      *     *     *        *
Seq 1: AATGGTGGGGA CTCAGATAAGACTGC AGGTTGATAAG TCGGAGGAAGGTGAGGATGA
Seq 2: CC GGCCGGGAACTCAAAGGAGAGACTGCCAG T GATAAACT GGAGGAAGGTGGGGATGA      1190

                               *   *        *  *    *  *
Seq 1: CGTCAAATCATCATCATGGCCCTTATGTCCTGGGCTACACGTGCTACAATGGC CTGTACA
Seq 2: CGTCAAGTCATCATGGCCCTTACGACCAGGGCTACACACGTGCTACAATGGGCGCA TACA      1249

        *
Seq 1: AAGCG>
Seq 2: AAGAGAAGCGACCTCGCGAGAGCAAGCGGACCTCATAAAGTGCGTCGTAGTCCGGATTGG      1309
```

FIG. 1C

```
Seq 1:  AAGCG>
Seq 2:  AAGAGAAGCGACCTCGCGAGAGCAAGCGGACCTCATAAAGTGCGTCGTAGTCCGGATTGG   1309

Seq 1:
Seq 2:  AGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGTGGATCAGAATGCCACG   1369
                                                               DDO3

Seq 1:                 DG73                    CACCC GAGTTGAGGATACCC
Seq 2:  GTTGAATACGTTCCCGGGGCCTTGTACACACCGCCCGTCACACCATGGGA GTGGGTTGCA   1428
                      DDO4                                     DDO2

Seq 1:        GAAGCTAT TATTCT AACCCGTAAGGGAGGAAGGTATTTAAGGTA TGTTT AGTG
Seq 2:  AAAGAAG TAGGTAG CTTAACCT TC GGGAGGGCGCT TACCACTTGTGATTCA TG   1482

Seq 1:  AGGGGGTGAA                                DG74
Seq 2:  ACTGGGGTGAAGTCGTAACAAGGTAACCGTAGGGGAACCTGCGGTTGGATCACCTCCTTA   1542
```
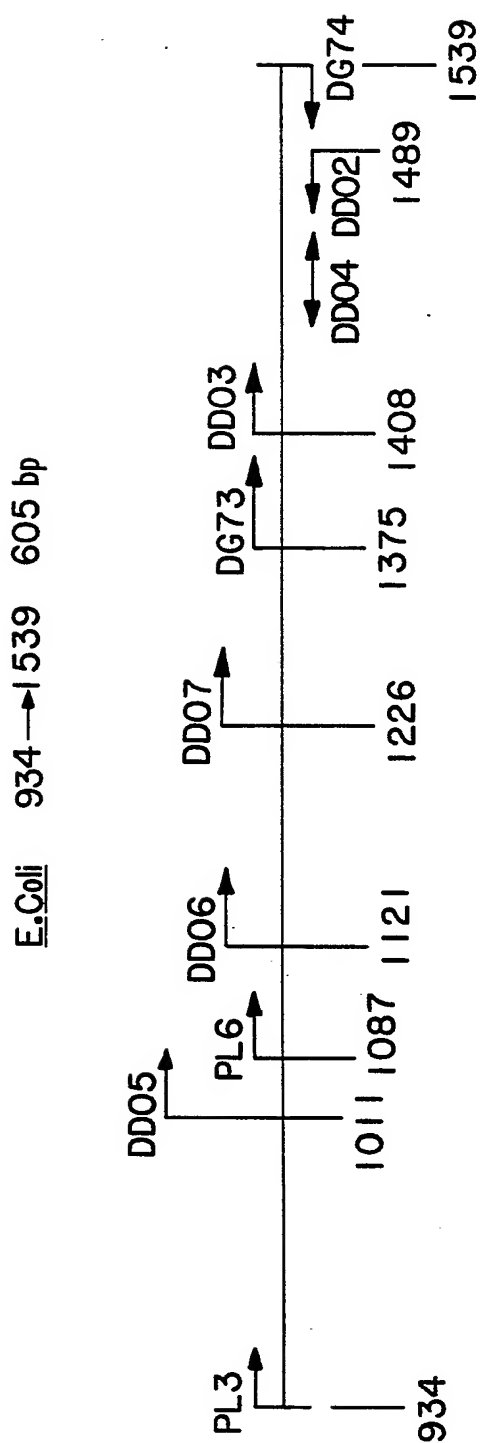
FIG. 2

E.Coli   934 → 1539   605 bp

PL3/DG74   934 → 1539
PL3/DD02   934 → 1489
PL6/DG74   1087 → 1539
PL6/DD02   1087 → 1489
DG73/DG74 1375 → 1539
DG73/DD02 1375 → 1489
DD03/DD02 1408 → 1489
DD03/DG74 1408 → 1539
DD05/DD02 1011 → 1489
DD06/DD02 1121 → 1489
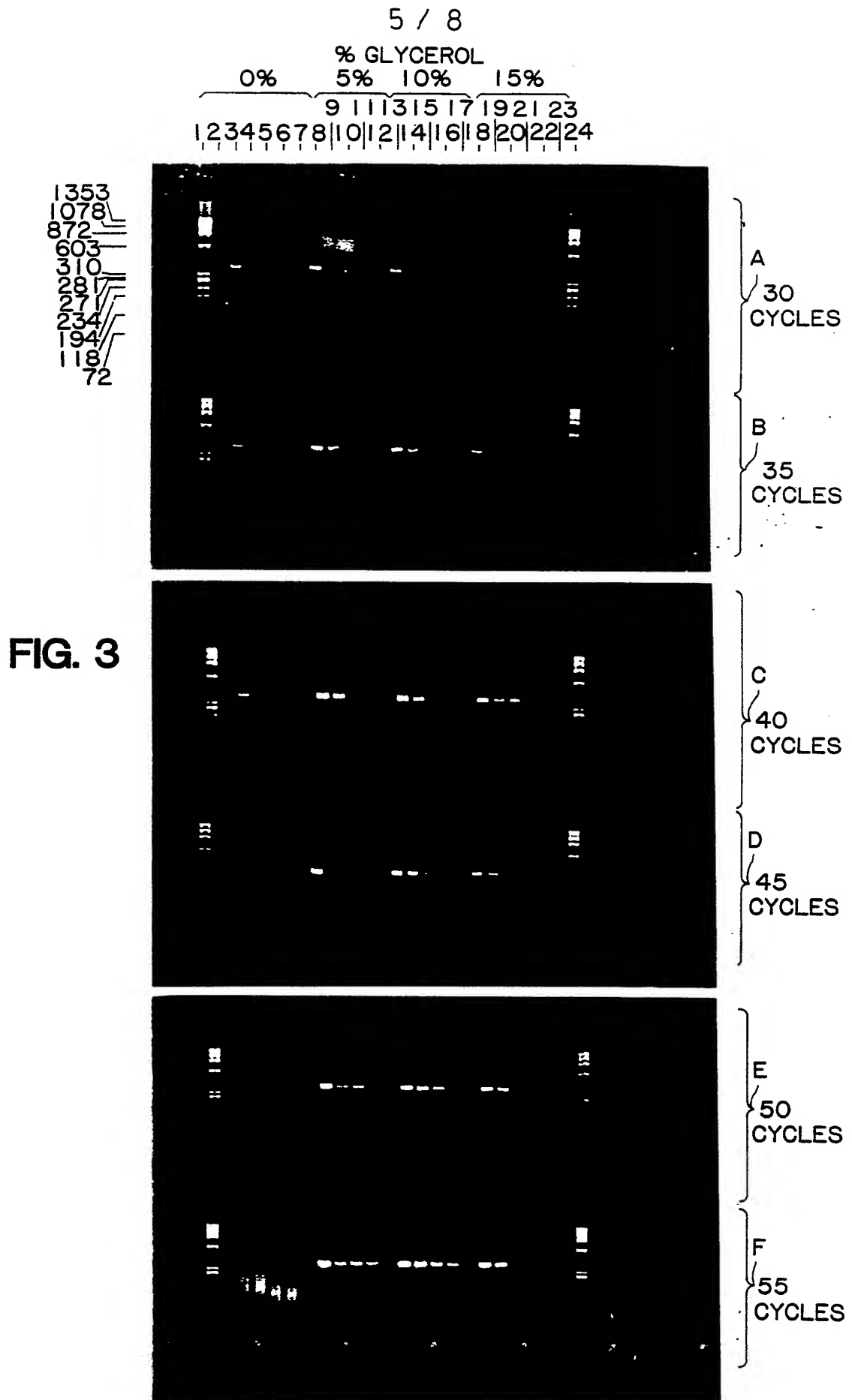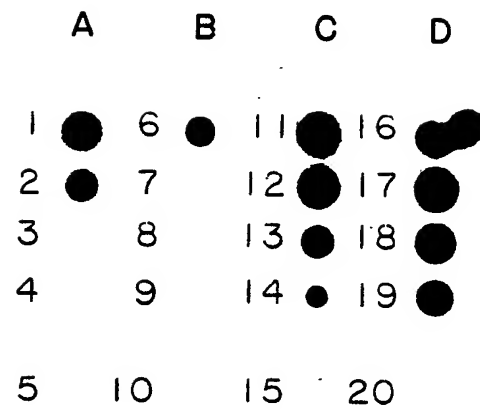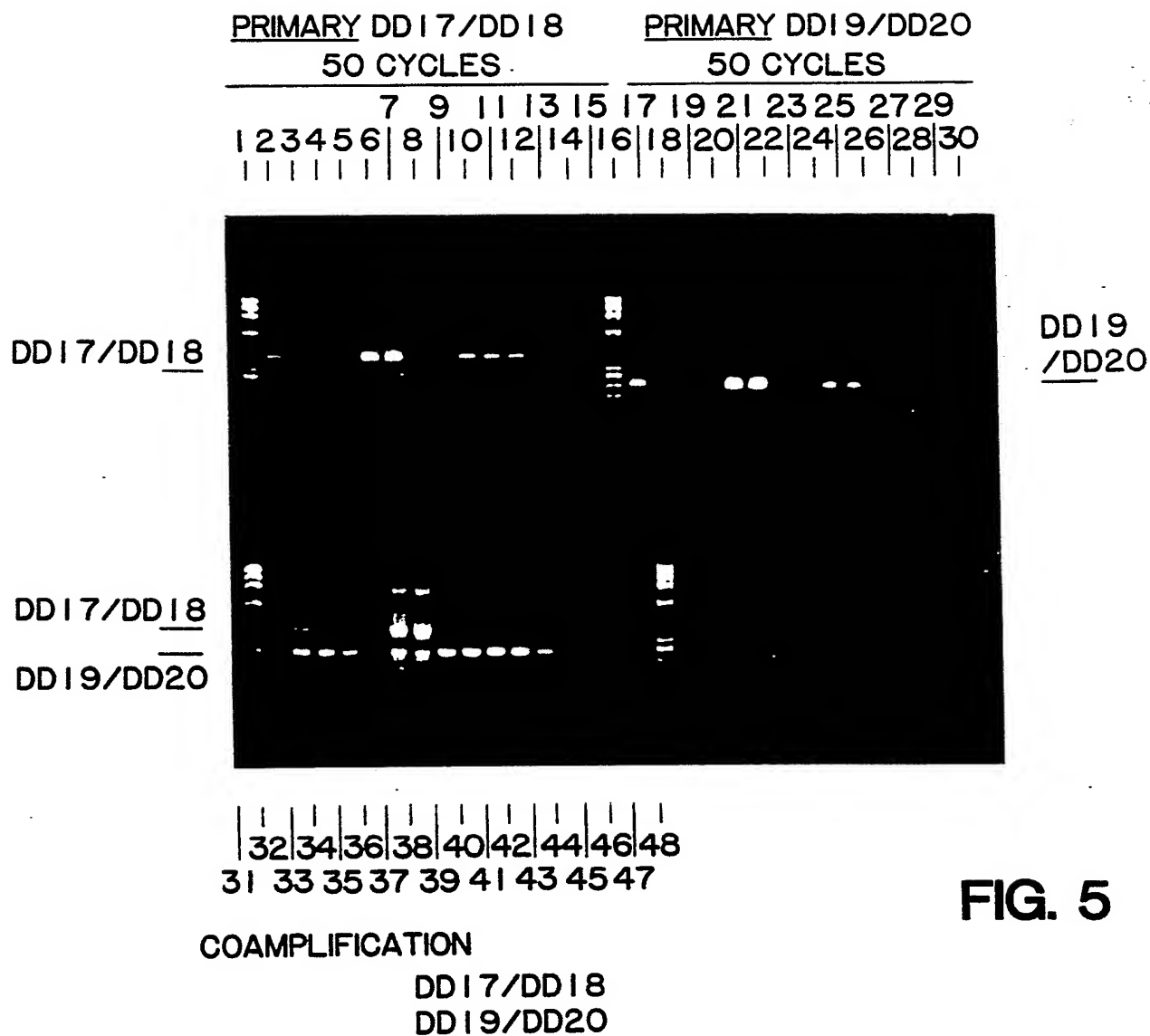DD07/DD02 1226 → 1489

5 / 8

FIG. 3

FIG. 4

PCR 46

PRIMARY DD17/DD18   PRIMARY DD19/DD20
50 CYCLES   50 CYCLES

DD17/DD18

DD19/DD20

DD17/DD18

DD17/DD18
DD19/DD20

COAMPLIFICATION
DD17/DD18
DD19/DD20

FIG. 5

```
                    DD06
B. burg    ATCTGTTACCAGCAGCATGTAATGGTGGGGACTCAGATAAGAGACTGCNNGNTNGATAAGTCGGAGG   62
B. herm    ATCTGTTACCAGCAGCATGTAATGATGGGGACTCAGACGAGACTGCNNGNTNGATAAG CCGAG     60
burg/herm  ........................A........CG......NN.N.N......C...

B. burg    AAGGTGAGGATGAGCGTCAAATCATCATGCCCTTATGTCCTGGGCTACACACGTGCTACA        122
B. herm    AAGGTGAGGATGAGCGTCAAATCATCATGGCCCTTATGTCCTGGGCTACACACGTGCTACA       120
burg/herm  ........................................................

                    DD25
B. burg    ATGGCCTGTACAAAGGGACAGAACAGTGATGTGAAGCAAACGGCATAAAGCAGGTCTCAG        182
B. herm    ATGGCCTGTACAAAGGCGATGAAACAGTGAAAACGGCATAAAGCAGGTCTCAG             180
burg/herm  ..........TGA....................................

B. burg    TCCGGATTGAAGTCTGAAACTCGACTTCATGAAGTTGGAATCGCTAGTAATCGTATATCA        242
B. herm    TCCAGATTGAAGTCTGAAACTCGACTTCATGAAGTTGGAATCGCTAGTAATCGTATATCA        240
burg/herm  ...A....................................................

B. burg    GAATGATACGGTGAATACGTTCTCGGGCCTTGTACACACCGCCCGTCACACCACCCGAGT        302
B. herm    GAATGATACGGTGAATACGTTCTCGGGCCTTGTACACACCGCCCGTCACACCACCCGAGT        300
burg/herm  ........................................................

                              DD04                            DD02
B. burg    TGAGGATACCCGAAGCTATTATTCTAACCCGTAAGGGAGGAAGGTATTTAAGGTATGTTT        362
B. herm    TGAGGATACCCGAAGCTATTATTCTAACCCGGAAGGGAGGAAGGTATCTAAGGTATGTTT        360
burg/herm  .......................................C................

B. burg    AGTGAGGG    370
B. herm    AGTGAGGG    368
burg/herm  ........
```

FIG. 6